ROLE OF Ki-67 AND p16 AS MARKERS OF PROGNOSTIC INDICES IN PREMALIGNANT AND MALIGNANT LESIONS OF CERVIX

DISSERTATION SUBMITTED TO

TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI

in partial fulfilment of

the requirements for the degree of

M.D. (PATHOLOGY)

BRANCH - III

TIRUNELVELI MEDICAL COLLEGE HOSPITAL

TIRUNELVELI

APRIL-2016

CERTIFICATE

This is to certify that this dissertation entitled **"ROLE OF Ki-67 AND p16 AS MARKERS OF PROGNOSTIC INDICES IN PREMALIGNANT AND MALIGNANT LESIONS OF CERVIX"** is the bonafide original work of **Dr. M. DINA MARY**, during the period of her Post graduate study from 2013-2016, in the Department of Pathology Tirunelveli Medical College & Hospital, Tirunelveli, in partial fulfilment of the requirement for M.D., (Branch III) in Pathology examination of the Tamil Nadu Dr.M.G.R Medical University will be held in April 2016.

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I hereby certify that this dissertation entitled **"ROLE OF Ki-67 AND p16 AS MARKERS OF PROGNOSTIC INDICES IN PREMALIGNANT AND MALIGNANT LESIONS OF CERVIX"** is a record of work done by **Dr. M. DINA MARY,** under my guidance and supervision, in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during her postgraduate degree course period from 2013-2016. This work has not formed the basis for previous award of any degree.

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DECLARATION

"ROLE OF Ki-67 AND p16 AS MARKERS OF PROGNOSTIC INDICES IN PREMALIGNANT AND MALIGNANT LESIONS OF CERVIX" submitted by me for the degree of M.D, is the record work carried out by me during the period of 2013-2016 under the guidance of **Dr. K. SHANTARAMAN M.D** Professor of Pathology, Department of Pathology, Tirunelveli Medical College, Tirunelveli. The dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, towards the partial fulfilment of requirements for the award of M.D. Degree (Branch III) Pathology examination to be held in April 2016.

Place : Tirunelveli
Date :

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PRINCIPAL INVESTIGATOR: Dr. M. DINA MARY, MBBS,

DESIGNATION OF PRINCIPAL INVESTIGATOR: POST GRADUATE IN PATHOLOGY
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Dear , Dr. M. Dina Mary, MBBS., The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 09.02.15.

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2. Study Protocol ☐
3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
5. Investigator's Brochure ☐
6. Proposed Methods for Patient Accrual Proposed ☐
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8. Insurance /Compensation Policy ☐
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10. Investigator's Undertaking ☐
11. DCGI/DGFT approval ☐
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ABBREVIATIONS

1.	HPV	-	Human Papilloma Virus
2.	CIN	-	Cervical Intraepithelial Neoplasia
3.	SCJ	-	Squamo-Columnar Junction
4.	IHC	-	Immuno Histo Chemistry
5.	LRIG 1	-	Leucine Rich Repeats and Immuno Globulin like Domains
6.	HER2	-	Human Epidermal growth Factor Receptor 2
7.	EGFR 2	-	Epidermal Growth Factor Receptor-2
8.	COX 2	-	Cycloxygenase-2
9.	ICMR	-	Indian Council Of Medical Research
10.	RAS	-	Rat sarcoma viral oncogene
11.	MICA	-	Micro invasive carcinoma
12.	DAB	-	Diamino benzedene
13.	AIS	-	Adeno carcinoma Insitu
14.	MIB-1	-	Molecular Immunology Borstel

CONTENTS

ABSTRACT

INTRODUCTION:

Since cervical cancer is highly attributed to the effects of HPV, an infectious agent, it can either be prevented or treated at a pre-invasive stage there by reducing morbidity and mortality ; hence the need for additional biomarkers and parameters of cell proliferation and cell death as important diagnostic and prognostic tools. This study was done to evaluate the proliferative indices in cervical pre cancerous and cancerous lesions i.e, mitotic count in hematoxylin and eosin sained sections and Ki-67 and p16 in IHC stained sections.

MATERIALS AND METHODS:

A total of 50 cases including CIN-1,CIN-2,CIN-3 and malignancy of the cervix were evaluated for mitotic figure count using light microscopy on H&E stained sections. Sections devoid of artefact were selected to count 1000 cells for mitotic figures and it was expressed in percentage. Strong nuclear positivity for Ki-67 was considered positive. The scoring was graded 1, 2, 3 for 10-30%,30-50% and > 50%. Staining intensity for p16 (nuclear or/and cytoplasmic staining) was done calculating the number of positive cells. The results were categorised as Grade 1,2,3 for 1-10%,10-50% and >50%positive cells.

CONCLUSION:

Ki-67 and p16 expression were seen in CIN-I,II,III and squamous cell carcinomas of the cervix.Ki-67 expression was in increasing grades from CIN to carcinoma. p16 had a better expression in high grade intra epithelial lesions (60-80%) and malignancies (100%)of the cervix compared to low grade dysplasias (21%). The mean mitotic count also showed an increase with increasing dysplasia. Thus Ki-67 and p16 expression in pre malignant and malignant lesions of the cervix can be used in conjunction with the histo morphological features to study their proliferative potential.

KEY WORDS: Ki-67, p16, CIN, Carcinoma cervix.

INTRODUCTION

Carcinoma cervix is a major burden on the population and the government especially in developing countries like India, although its incidence has been controlled in developed nations. This decrease may be attributed to the efficacy of cervical cytology screening programmes which are done in a categorical manner.

Carcinoma cervix stands next only to breast cancers that affect the female population being responsible for about 5% of deaths due to malignancies worldwide[1]. The estimated incidence of cervical cancer is 470,000 and remains as a leading cause of morbidity and mortality worldwide[2]. Approximately 230,000 women die each year from cervical cancer; over 190,000 of these women are from developing countries. The contribution is roughly around 25% by the Indian women to the total occurrence in the world.[3]

Biopsy cervix is definitive in establishing a diagnosis in cervical lesions. However, it has its own limitations such as the study of morphological parameters alone with no information as to the fate of the lesion (progression / regression). Further inter observer variability needs special mention.[4,5] Hence to overcome these draw backs and to increase the accuracy in deciding the magnitude of progression, bio markers come into role.

The carcinoma cervix has histopathologically, well characterized precursor lesions (CIN), which slowly progresses to the well differentiated tumour. The vast majority of HPV infections (up to 90%) regress spontaneously, without treatment,

after a few months. If the viral infection persists, however, the risk of developing a precancerous lesion increases as well as the risk of developing an invasive carcinoma.[6] This transformation of cervical epithelial cells from CIN to carcinoma takes 10 – 15 yrs.[7] During this transformation period , many important markers of tumor progression, are expressed.

Carcinoma cervix, thus because of its long period of transformation, provides a great opportunity to study about the expression of biomarkers of tumor progression. Because of the above advantages, present study is designed to evaluate the mitotic count and the expression of immunomarkers, Ki-67 and p16 in different grades of cervical precancerous and cancerous lesions to understand their role in cervical carcinogenesis.

AIMS AND OBJECTIVES:

1. To collect data on occurrence of cervical pre malignant and malignant lesions reported at TVMCH.

2. To evaluate the proliferative indices in cervical pre-cancerous and cancerous lesions - mitotic count in haematoxylin and eosin sections and Ki-67 and p16 in IHC stained sections.

3. To correlate the proliferative indices with varying grades of pre malignant lesions and various histological sub types of cervical carcinoma.

REVIEW OF LITERATURE

EPIDEMIOLOGY OF CANCER CERVIX:

Cervical cancer is one of the most common cancers among women worldwide (WHO 2009). Majority of cervical cancer cases today occur in the developing world.

Approximately around 5 lakhs new cases of carcinoma cervix occur worldwide of which nearly 50% culminate in death.[1] The incidence rate has declined in both white and African American women, for the past few decades. Since 2004, rates have decreased by 2.1 % per year in women younger than 50 years of age and by 3.1% per year in women aged 50yrs and above.

The Indian sub-continent has a considerable population of females in the reproductive age group who at risk of developing cervical cancer. According to the current statistics, around 1.25 lakh new cases that are diagnosed and 75000 deaths in our country are due to cancer cervix.[2] According to National Cancer Registry of ICMR the incidence in India is 14.42/100000 population with mortality rate 2.83/100000 population (ICMR 2004).

ANATOMY OF CERVIX:

The cervix (term taken from the Latin, meaning neck) is the continuation of the body of the uterus. The cervix is anatomically divided into portio vaginalis that protrudes into the upper vagina and supravaginal portion. It has an anterior and a posterior lip. It is 2.5–3 cm in length in the adult nulligravida. In the anatomical position, it has a slight angulation.

The portio vaginalis of the cervix is divided into three parts- endocervix, ectocervix, and the transformation zone.

The Ectocervix is the portion of cervix which extends from the squamo - columnar junction to the vaginal fornices.

The Endocervix is the portion of cervix which extends from the internal os to the ectocervix and it has the endocervical canal.

The Squamocolumnar Junction (SCJ) denotes the region where the squamous epithelium of ectocervix and columnar epithelium of the endocervix meet.

HISTOLOGY OF CERVIX:

The cervix has both columnar and squamous epithelial lining with the sub epithelium composed of fibromuscular tissue with blood vessels which gives it the tough consistency. Smooth muscle occupies mainly the endocervix and comprises 15% of the substance.

Ectocervix:

The ectocervix is lined by mature nonkeratinizing stratified squamous epithelium.

It has three zones:

1) Basal /parabasal cell layer, which is responsible for the continuous epithelial regeneration, hence otherwise called germinal layer

2) Stratum spinosum, which lies between the basal and the superficial layer forms the major portion

3) Superficial zone, composed of mature cells

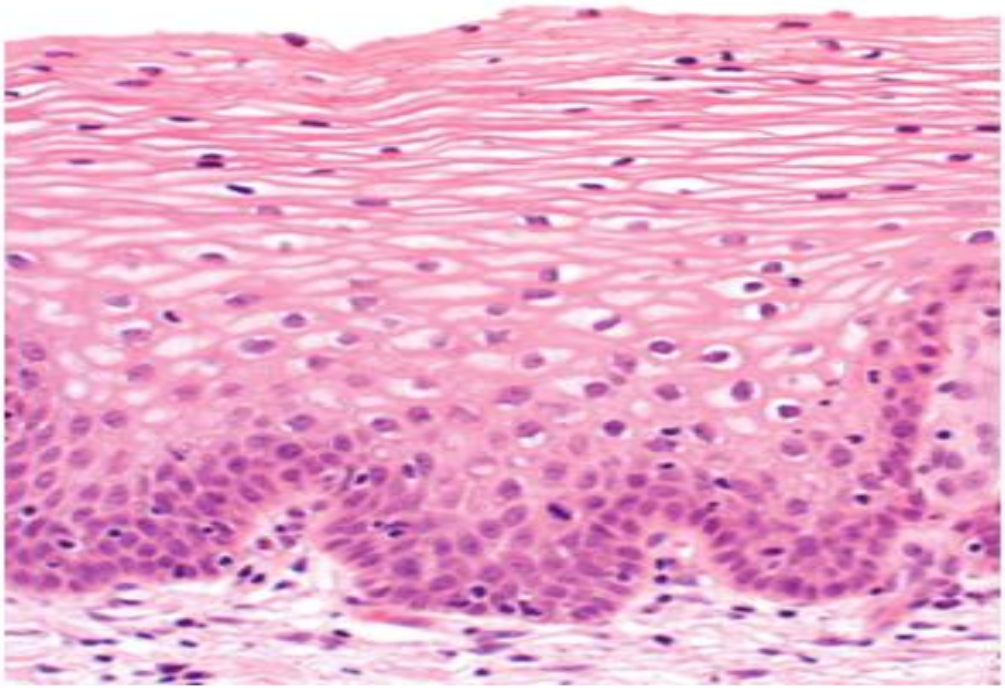

Fig. 1. Histology of ectocervix showing orderly maturation

The basal layer has two types of cells. The basal cell, which is about 10 nm in diameter, with very minimal cytoplasm and elongated nuclei placed perpendicular to the basal membrane which is beneath it.

The parabasal is called so because of its histomorphological position. Parabasal cells which are one-to-two-cells thick over the basal epithelium, are larger having more cytoplasm. These cells are mainly concerned with continuous regeneration.

The midzone is occupied by cells undergoing maturation, characterized by a gradual increase in the volume of the cytoplasm but the nuclear size, however, remains the same up to the most superficial cell level. Once exfoliated these cells are referred to as the intermediate cells. These cells do not undergo cell division. Intermediate cells have abundant intracellular glycogen, responsible for the clear, vacuolated appearance of their cytoplasm.

The most differentiated compartment of the squamous epithelium is the most superficial portion composed of flat cells. The cells in this portion have abundant cytoplasm with condensed nuclei.

During the reproductive period the epithelium of the ectocervix is under continuous remodelling by proliferation, maturation, and desquamation. The epithelium takes 4-5days for a cycle of regeneration to complete.

Endocervix:

The endocervical canal and the underlying glands are lined by mucin-secreting, columnar epithelium. The columnar epithelial cells have granular cytoplasm filled with mucin and basally placed oval nuclei.

The subepithelium of the endocervical mucosa has a well-developed capillary network .True lymphoid follicles with or without formation of germinal centers, are occasionally seen in the subepithelial stroma of both the ectocervix and the endocervix.

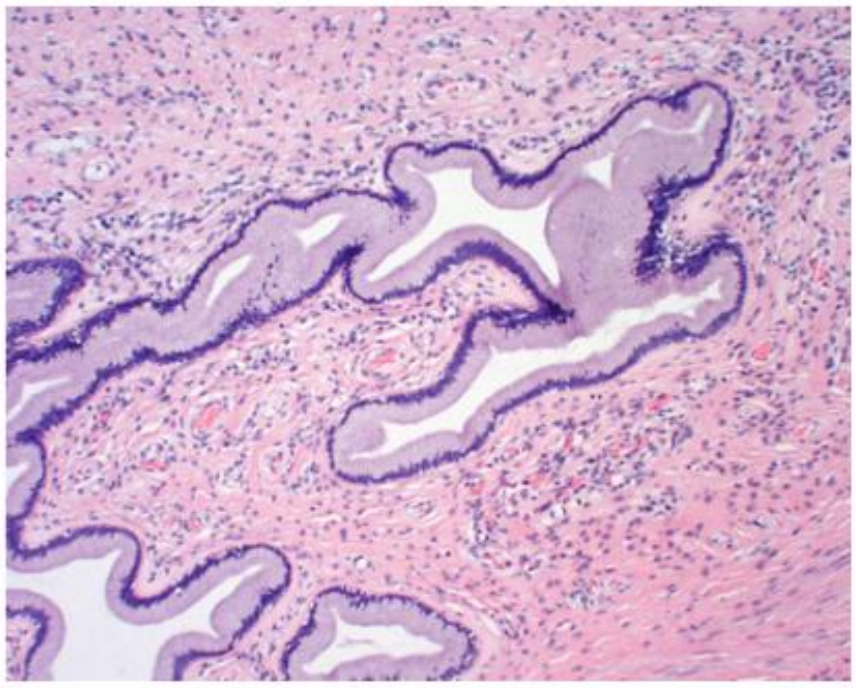

Fig.2. Histology of endocervix showing glands lined by mucinous epithelium

There is another kind of ciliated cells, which are non secretory in nature present in the endocervix. The main function of these ciliated cells is that they help in distribution and mobilization of the endocervical mucus.[8] The other types of cells that are seen in endocervical epithelium include argyrophil, neuroendocrine and argentaffin cells.[9] These cells can be demonstrated by histochemical stains

Transformation zone:

The transformation zone is the junction between the stratified squamous epithelium of the ectocervix and the glandular epithelium of the endocervix. This is otherwise called the squamo columnar junction. This is constantly subjected to hormonal influences, and as a consequence, its anatomic location keeps varying with age.

The importance of transformation zone is that it withstands the most important function of differentiation. This zone includes cells destined for both squamous and columnar cell differentiation. These changes /differentiation develops in response to inflammatory stimuli and alterations in pH that occur following the onset of menarche. They may be identified by basal/reserve cell markers. A generic biomarker that identifies all of these cells is p63.

Basal and columnar cells appear most susceptible to infection, based on the requirement that these cells be exposed to the virus for infection to take place. Presumably these cells contain receptors targeted by the papillomavirus.

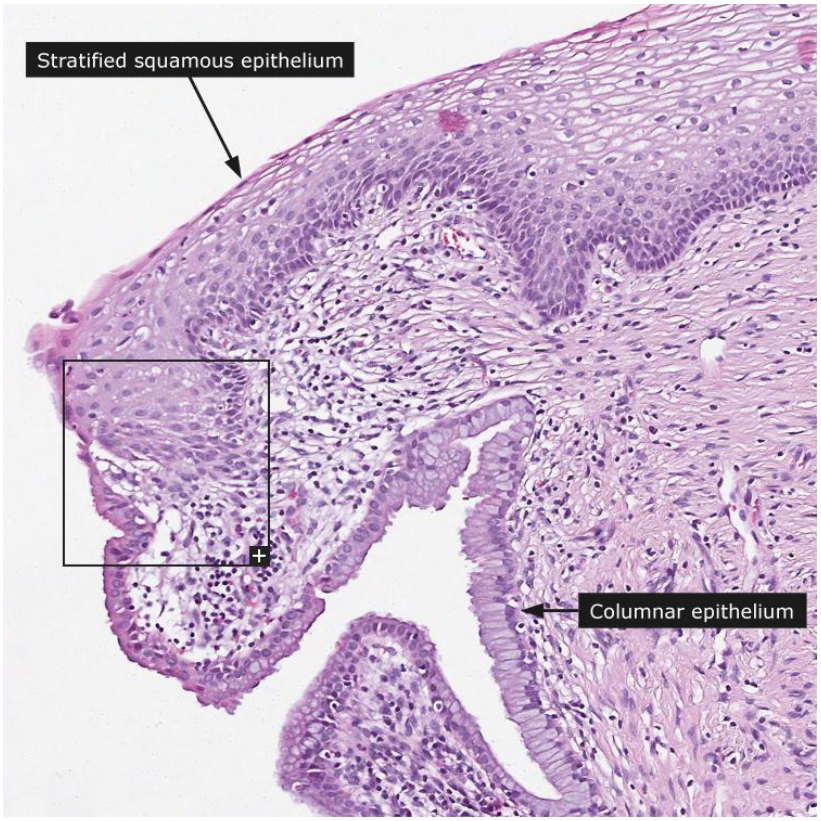

Fig. 3. Picture showing microscopic appearance of squamo columnar junction

At birth, female neonates have endocervical epithelium present in the portio which is because of intrauterine exposure to maternal hormones. But this rapidly keeps regressing into the endocervical canal until menarche. During puberty, pregnancy, or progesterone therapy, the presence of endocervical glandular epithelium in the ectocervix results in what is clinically known as physiologic cervical eversion or ectropion.

During the reproductive years, the endocervical epithelium is constantly replaced by metaplastic squamous epithelium. This is due to exposure of the ectropion to the acidity of the vagina and other environmental factors. The degree

of ectropion begins to decrease with increasing age and time from onset of sexual activity.

During menopause, this zone recedes, and in postmenopausal women may be located completely within the endocervical canal. Hence, the transformation zone is the remodelled area of ectropion which undergoes active squamous metaplasia and thereby represents the region between the original and the functional SCJ.

The transformation zone is a very dynamic area, which keeps changing under hormonal and environmental influences. Remodeling of the ectropion is not always in a uniform fashion, and, in fact, squamous metaplasia can, and often does, occur anywhere within the exposed endocervical columnar epithelium in a patchy fashion. This area is the one most susceptible to HPV infection for several reasons, including higher susceptibility of the advancing edge of the immature squamous epithelium to infection.

CERVICAL INTRA EPITHELIAL NEOPLASIA:

It was during the beginning of this century that the existence of precursor lesions of carcinoma cervix was extensively studied. Sir John Williams was the first to comment on dysplastic changes in the epithelium seen in cases of frank invasive carcinomas in 1886.This was followed by description of the histological appearance of these intra epithelial lesions which had histological features similar to the adjacent invasive component.

The recognition of many similarities between the cervical intra epithelial lesions and invasive squamous cell carcinoma led to confirmation of the hypothesis that invasive squamous cell carcinoma develops from a defined intra epithelial lesion[10]. To add strength to this fact, many of the long-term follow-up studies, clearly demonstrated that a significant proportion of patients with carcinoma in situ who were left untreated presented with invasive squamous cell carcinoma.

The World Health Organization (WHO) first proposed unified terminology to define and report cervical carcinoma precursor lesions in cervical biopsy specimens. Intra epithelial squamous dysplasia was thereby defined as a "lesion in which part or whole of the thickness of the epithelium is replaced by cells showing varying degrees of atypia, and it was further divided into mild, moderate, and severe.

Even then, there was no widely accepted criteria for separating the grades of dysplasia and the further separation of each lesion into one of the categories was highly subjective. The separation of dysplasia from carcinoma in situ implies two distinct disease processes rather than a spectrum of severity of what is now known to be a single disease process.

The terminology of CIN was similarly divided into three categories, CIN I, CIN II, and CIN III, with carcinoma in situ being incorporated into the CIN III category. Carcinoma in situ was further subdivided by some authors into parabasal

cell, keratinizing cell, pleomorphic cell , and small cell types which is of no significance.[11,12]

The vocabulary of cervical intraepithelial lesions supposed to represent the precursors of invasive carcinoma, has progressed over the years and continues changing today.

The basic principles are the following:

1. Nearly all invasive carcinomas of the cervix are preceded by a stage in which the atypical cells remain confined to the epithelium (intraepithelial stage).

2. These intraepithelial lesions share many of the cytologic features of the invasive carcinoma, which include nuclear enlargement, irregular nuclear membrane, hyperchromatic nuclei; increase in mitotic activity; and disorderly maturation pattern. There is also a diminution or absence of cytoplasmic glycogen which can be illustrated by the reduction or lack of staining with the Lugol iodine or Schiller test.

3. A series of pattern of morphologic abnormalities among these lesions provide a rough indication of the possibility with which they will turn into invasive carcinoma, if left untreated. These morphologic abnormalities inturn show correlation with immunohistochemical, cytogenetic, cellular proliferation, DNA ploidy and molecular changes.

4. It has been proven that in the large majority of cases, the process particularly affects the areas of squamous metaplasia located at the transformation zone and in its endocervical side and sparing the native squamous epithelium of the ectocervix.

5. The microscopic criteria for the diagnosis of these lesions remains the same regardless of the situations.

The lesions induced by HPV in the cervix can range anywhere between a condyloma to frank malignancy. Exophytic condylomata are less common in the cervical transformation zone. They are associated usually with HPV types 6 and 11.

These lesions exhibit acanthosis, verruciform growth pattern, and viral cytopathic effect (koilocytotic atypia).[13] The verrucous pattern is typified by blunt papillae. The superficial atypia is characterized by karyomegaly, nuclear enlargement with binucleation, irregularities in nuclear membrane, and hyperchromasia.

Metaplasia refers to replacement of one differentiated cell by another cell type. Though it serves as a protective mechanism to withstand tough conditions it may provide the initiative towards neoplasia. Likewise columnar epithelium of the endocervix also undergoes metaplasia to a stratified squamous epithelium.

This makes the epithelium particularly vulnerable to infections by the viruses, and other risk factors leading onto the development of an intraepithelial

neoplasm. Differences in the differentiation level or differentiation pathway of the underlying mucosa may influence the morphologic presentation of cervical lesions, leading to additional patterns not accounted for in traditional classifications.

The impact of HPV infection on squamous epithelium can be summarized in two important histologic parameters:

1. Alterations in the density of superficial epithelial cells. The persistence of a higher nuclear density manifested by either more nuclei per unit area or more nuclear area.

2. Superficial squamous atypia. In mature SILs, the effects of HPV infection produce nuclear enlargement, variations in nuclear size, and binucleation.

The HPV induces a variety of squamous pathologies which can be a minimal lesion like flat condyloma to a highly invasive carcinoma. Flat condyloma, is a plaque lesion involving ectocervix with the transformation zone, with koilocytosis as the cardinal finding. The koilocytic atypia is recognized by cytoplasmic vacuolation[14] around the nuclei with condensation of the cytoplasm at the periphery, nuclear atypicality include nuclear enlargement, hyperchromasia, chromatin clumping, marked nuclear membrane irregularities, and multinucleation. Condyloma can coexist with low or even high- grade CIN.

Table 1 : Older and Newer WHO terminology of CIN

Older classification	WHO classification
Mild dysplasia	CIN I
Moderate dysplasia	CIN II
Severe dysplasia/carcinoma in situ	CIN III

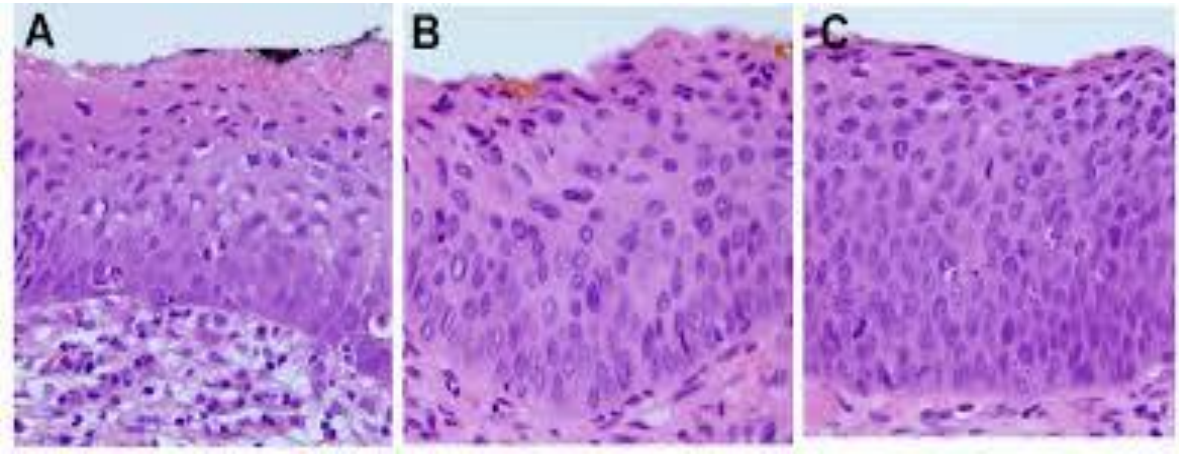

Fig.4. Microscopic appearance of progressive stages: A-CIN-1,B-CIN-II and C-CIN-III

The morphologic spectrum of CIN to frank carcinoma can be seen as a continuum.

CIN 1 (mild squamous dysplasia) is defined as the presence of dysplastic squamous cells in the lower third of the epithelium along with HPV changes in the rest of the superficial epithelium. The presence of mitotic figures several cell layers above the basal membrane supports a diagnosis of CIN 1. The presence of atypical mitosis even at the basal level also attests the presence of dysplasia.

The features of LSIL in mature squamous epithelium include the following:

1. Conspicuous superficial cell atypia with binucleation, two-fold nuclear enlargement, and variable nuclear chromasia.

2. Generally low N/C ratio in maturing epithelial cells with preserved cytoplasmic differentiation.

3. A subtle expansion of the lower third of the epithelium, signifying a mild delay in epithelial cell maturation.

4. A range of nuclear features in the lower third of the epithelium that include either euchromasia or uniform hyperchromasia, with minimal variation in nuclear size and shape. Enlarged nuclei in the lower layers usually have minimal chromatin complexity and reside within cells undergoing cytoplasmic maturation.

5. Generally well-preserved polarity with uniform transitions to mature epithelium. Exophytic lesions associated with HPV-6/11 will exhibit patchy or negative p16 staining.

Most flat lesions will exhibit diffuse p16 staining in the lower epithelial layers.

CIN 2 (moderate dysplasia) is defined by presence of dysplastic squamous cells in the basal two-thirds of the epithelium; the remaining of the epithelium shows some differentiation with maturation, with, as in CIN 1. Nuclear abnormalities are more marked than in CIN 1, and more nuclei with greater degrees of abnormality are seen high in the epithelium. The diagnosis of CIN-II is

highly subjective to inter observer variability,[15] but however its differentiation from CIN-I is clinically mandatory.

CIN 3 (severe dysplasia) is defined by dysplastic squamous cells marked throughout the whole thickness of the epithelium. Mitotic figures which are usually confined to the basal layers are found at all levels of the epithelium and may be numerous, with many abnormal configurations. The findings in the upper portion of the epithelium include more extensive nuclear changes. CIN-II and CIN-III are together considered high grade intra epithelial lesions.

The features can be summarised as follows:

1. In general, less maturation, higher nuclear density, and a less orderly transition from the immature to mature epithelial layers.

2. Abnormal cell differentiation

3. Greater differences in nuclear size and staining as well as contours and chromasia in the lower epithelial layers. In essence, these features reflect the effects of oncogenic papillomaviruses on the biology of the replicating (lower epithelial) cell layers.

4. In addition to a broader distribution of nuclear atypia and loss of cell polarity, these lesions are distinguished to some degree from LSIL by increased mitotic index and abnormal mitotic figures. CIN-3 lesions (including carcinomas in situ) contain full-thickness nuclear atypia. However, the degree of variation in nuclear size, contour, and staining

may vary, giving rise to variants of CIN-3 that closely mimic immature metaplastic epithelium, with more subtle degrees of karyomegaly or anisokaryosis. Such cases may be difficult to recognize on both cytologic and histologic examination, and other strategies, such as the use of biomarkers, may be required in such cases.

5. Changes usually attributed to the presence of HPV infection, such as koilocytosis and epithelial multinucleation, are often present and are most conspicuous in CIN1 and 2,and minimal or absent in CIN3.This may be a reflection of viral integration only in the high grade lesion.

High-grade cervical intraepithelial lesion should be distinguished from atypia of repair, radiation changes, atrophy, immature squamous metaplasia, transitional metaplasia and invasive squamous cell carcinoma.

In atypia of repair, the squamous epithelium may be disorganized, atypical basal-like cells may be present up to the mid-zone, and there may be nuclear atypia with nuclear enlargement causing confusion with CIN.

However, in repair some maturation of the squamous epithelium towards the surface is made out with the cells having well-defined cell borders that show no crowding. Further there is no variation in cell size or shape. There is no coarse chromatin but prominent nucleoli, and if cytoplasmic halos are present, they are small and uniform secondary to accelerated cell maturation.

Frequently, there is acute inflammation, spongiosis, and mitoses that are on fined to the parabasal layers of the epithelium. Ki-67 can be helpful in distinguishing SIL from reactive epithelium as staining should be confined to the lower third of the epithelium in atypia of repair.

In cases where reactive changes can rarely coexist with CIN, a point in favour of the diagnosis may be the finding of nuclei with HPV effect in the superficial layers.

Radiation changes can be distinguished from CIN both by an increase in the nuclear size and the amount of cytoplasm. Therefore, there is a proportionate or decreased nuclear-to-cytoplasmic ratio. Nuclear spacing is uniform with minimal nuclear crowding and the chromatin is smudgy, rather than coarse. Mitoses are rare and cytoplasmic vacuolization may be present.

Atrophic squamous epithelium may be seen in post- menopausal women or those on Depo-Provera. Distinguishing atrophy from CIN can be very difficult as in cases where the epithelium lacks maturation; the cells have increased nuclear-to-cytoplasmic ratio; and the nuclei are small with coarse and hyperchromatic chromatin. However, in atrophy, the nuclei are uniform in size and spacing, and there is minimal nuclear pleomorphism and absent to rare mitoses Ki-67 can be helpful in this distinction, as atrophy should show minimal to absent staining, while CIN shows strong positive staining involving at least the upper two- thirds of the epithelium.

Immature squamous metaplasia can also be difficult to distinguish from CIN, as immature squamous cells with increased nuclear-to-cytoplasmic ratio may occupy almost the full thickness epithelium, and maturation may only be seen towards the surface. However, there is no cell crowding and, unlike CIN, cell membranes are usually well defined. The nuclei are uniform in size and shape, they have fine chromatin, and small nucleoli may be seen. Even though mitoses may be present, abnormal mitoses are lacking. Columnar cells may be present on the surface overlying the metaplastic epithelium but are only rarely present overlying dysplastic epithelium, and thus is a helpful feature.

Differentiating atypical immature squamous metaplasia from CIN is even more difficult and sometimes not possible. The degree of nuclear atypia is usually less prominent than HSIL and although mitotic figures may be present, they are not abnormal, as they can be in HSIL. p16 immunohistochemistry may be useful in some of these cases.

Transitional metaplasia is seen predominantly in post- menopausal women where it can involve the transformation zone, the ectocervix as well as the vagina. It can be misdiagnosed as high grade CIN since this lesion is more than ten cell layer in thickness with absence of maturation and elongated nuclei exhibiting irregular nuclear contours. The differentiating features include very occasional mitoses, horizontal orientation of the cells in the superficial layer which show streaming and oval nuclei with fine chromatin and grooving. However, this condition can be present in conjunction with dysplastic changes.

RISK FACTORS ASSOCIATED WITH CIN AND CARCINOMA CERVIX :

The risk factors associated with the development of carcinoma cervix can be broadly divided into two:

1. Viral
2. Non-viral

The non-viral factors include:

1. Age
2. Immune status
3. Multiple sexual partners
4. Oral contraceptives
5. Other associated infections

Virus as risk factor:

The first human cancer to be found to have infectious agent as a causative factor is the cervical cancer. (Thomson et al 2008.) The human papilloma virus infection is the most important risk factor of cervical cancer[16]. Almost all of the invasive cervical cancers are preceded by cervical intraepithelial neoplasia (CIN) induced by HPV. Not all HPV related lesions present as condyloma or low-grade lesions; high-grade lesions may appear at any point of the infection depending on HPV types and quantity.

Human papilloma virus has nearly 150 types which are classified into high risk, probable high risk, and low risk[17].

The low-risk subtypes include HPV 6, 11, 42, 43, 44, and 53.

The High- risk subtypes include HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The high risk type of HPV and its persistence is the most important of all.[18] However, viral load will not discriminate low- from high-grade CIN because viral production is often higher in low-grade lesions.

The human papilloma virus enters the basal cells or immature squamous metaplastic cells through defects in the mucosa at the transformation zone. It usually infects the squamous epithelium; however, the virus can also infect subcolumnar reserve cells.

The virus can induce either a latent or a productive infection. In the productive infection, large amounts of free DNA virus are produced in the intermediate and superficial cell layers, which are considered nonproliferating terminally, differentiated squamous cells. As the virally infected cells mature and move towards the surface, the characteristic cytopathic effect – the so-called koilocytic atypia – becomes apparent.

Incorporation of HPV DNA into the host cell genome, with covalent binding of viral genome into host DNA, is considered a critical event in the progression to high-grade cervical intraepithelial lesions.

High-risk HPVs induce changes in the epithelium by producing E6 and E7, two proteins that have significant growth-stimulating and transforming properties. As the virus gets integrated with the host genome, there is enormous expression of

E6, E7 due to disruption of the viral DNA[19]. This excess in the levels of E6/E7 proteins leads to down regulation of p53/Rb which are tumor suppressor proteins with the end result being uncontrolled cell proliferation with dysplastic changes.[20,21]

It has been found in various studies that low-risk HPV is almost always associated with low-grade cervical intraepithelial neoplasia which frequently regresses, and its association with cervical carcinoma is exceedingly rare, whereas high-risk HPV subtypes can result in either low or high grade lesions. Moreover, if left untreated, it subsequently progresses to invasive carcinoma.

HPV-16 is detected in nearly 50% of HSIL and squamous carcinomas of the cervix and is the "prototypic" cancer-causing virus. HPV-18 is associated with less than 15% of squamous carcinomas, predominating in adenocarcinomas in situ and invasive adenocarcinomas.

HPV testing has been approved for cervical cancer prevention, keeping the following in mind:

HPV testing combines high sensitivity and an objective measurement of cervical cancer or precursor risk, and the increase in risk attending its presence is approximately 40-fold.

- Combined negative HPV testing and normal smear may increase the duration of the "protective effect." Because HPV is detected prior to the development of a cytologic abnormality, it is likely that patients

negative by both HPV testing and smear may be followed at longer intervals than by smear alone.

- The index of HPV positivity drops markedly over age 30, and this group has been selected for screening. The degree to which an HPV test is more specific for neoplasia risk in this group varies according to the study.

- A significant fraction of menopausal women may score positive for HPV, A number of studies have emphasized the importance of HPVs 16 and 18, the two most prevalent HPVs in cervical cancer

Non-viral risk factors:

Age :

Young, sexually active women are at greatest risk for HPV infection and preinvasive cervical neoplasia. This risk gets reduced significantly with increasing age, which is associated with decreasing risk of cancer. The risk is reduced with the attainment of menopause. The progressive drop in risk with increasing age can be attributed to an effective immune response against the virus that follows the onset of sexual activity and exposure to HPVs. Protection is long lasting, given the low rates of HPV positivity in middle-age women.

HLA type:

HLA that belongs to class II haplotypes (linked class II alleles) are generally related to cervical intraepithelial neoplasia and invasive carcinoma.

Among this certain class II alleles are related to LSIL, HSIL, and full blown malignancy, but certain other type II alleles are protective in nature.[22] Still others have been associated with infection alone or certain HLA types are associated with cancers produced by certain HPV types, such as type 16. The studies observed that specific HLA class II haplotypes may influence HPV antigen presentation and the immune response to HPV infection, in turn influencing the risk of developing invasive cervical carcinoma.

Immune status :

Immunodeficiency conditions like HIV infection, post transplantation,[23] those on immunosuppression drugs have increased risk of developing cervical carcinoma. The reason is there is deficient cell mediated immunity and therefore increased risk of cervical cancer.[24] The impact of HIV infection on cervical cancer risk is controversial, but prognosis appears worse in patients with severe immunodeficiency.

The risk of cervical neoplasia or HPV infection in immunosuppressed individuals is well documented. Cancer rates are higher in this population, and in general anogenital cancers occur at a younger age.

HIV-infected individuals are particularly prone to persistent HPV infection, and a higher risk of precursor lesions. The prognosis of cervical cancer in HIV infection appears to be worse due to severe immunodeficiency. The rate of the virus infection in the immunosuppressed patients were found to be increased 9 folds when compared to the general population.

The risk of HPV positivity is increased in women who are HIV positive[25] Approximately 60% of HIV-infected women versus 36% of uninfected women score positive for HPV, based on testing of cervical samples. Frequencies of HPV-16, HPV-18, and multiple infections are also significantly more common in the HIV-infected group. The risk of a subsequent squamous intraepithelial lesion is also significantly higher in HIV-infected women.[26]

Multiple sex partners:

It has been proposed that if the male partners have multiple sexual partners, it influences the risk of cancer development, even though it is of lesser degree. Krebs and Helmkamp showed that treating the male partner did not influence risk of recurrent genital warts. The implication of this study is they are not reinfected by the same virus, which is in consistence with a functioning immune system. Condoms protect against HPV infections, but incompletely. Circumcision reduces the risk of infection in the male partner and his female contacts. Moreover, timing of sexual activity gains more importance than absolute number of sexual partners in conferring risk of current HPV infection.

Oral contraceptives:

The use of oral contraceptives has shown to have increased risk for the development of carcinoma cervix. The strong theoretic basis for this is the hormones influencing epithelial growth which increases the susceptibility to neoplasia in the cervical transformation zone[27].

Oral contraceptive use with their plasma levels has a linear association with cervical neoplasia[28]. Indeed there is a strong association between hormonal replacement and cervical adenocarcinoma. A recent meta-analysis postulated a relative risk of 1.90 with use of oral contraceptive pills for greater than 5 years and a decline in risk with cessation of use.[29].

Smoking:

Smoking increases the risk of cervical neoplasia, however the mechanism is unclear. The reason behind this is presumably the presence of DNA adducts in the cervical mucus, thereby exposing the transformation zone mucosa to carcinogens.[30] Genetic polymorphisms that theoretically do not reduce adduct formation have also been implicated. There is also a dose- response relationship between the two.

Other associated infections:

The association between chlamydia infection and cervical neoplasia is controversial. There is evidence that infection with chlamydia serotype G confers a significant risk of cervical cancer.[31] There are several studies showing a relationship between genital infections and HPV or CIN.

In summary, a multitude of factors, viral and host related, influences risk of cervical neoplasia before, during, and following exposure and lesion progression.

CARCINOMA CERVIX:

The World Health Organization (WHO) divides invasive carcinoma of the cervix into three categories :

- squamous cell carcinoma
- adenocarcinoma and
- other epithelial tumors

The ‘‘other epithelial tumors’’ include

- adenosquamous carcinoma
- adenoid basal cell carcinoma
- adenoid cystic carcinomas
- neuroendocrine tumors and
- undifferentiated carcinoma

Squamous cell carcinoma of cervix is the most common histological variant followed by adenocarcinoma and adenosquamous carcinoma.

Precautions to be considered before reporting carcinoma cervix:

1. Excluding non-neoplastic infections, such as herpes or chlamydia
2. Understanding the transformation zone and the plethora of benign alterations in epithelial differentiation that characterize this region
3. Applying the criteria for differentiating grades of CIN

4. Understanding the influence of transformation zone epithelial subsets on the morphology of type-specific infections in the transformation zone, producing patterns that do not conform to classic descriptions and less easily classified lesion patterns

5. Having a working knowledge of the differential diagnosis of classic and variant forms of SIL

MICRO INVASIVE SQUAMOUS CELL CARCINOMA:

This is also called early invasive carcinoma of cervix. This tumor lies in the spectrum between CIN and well established invasive carcinoma. This is defined as tumor less than or equal to 3mm in depth and 7mm in length without capillary or lymphatic invasion. Practically almost always associated with high-grade CIN, these are small irregular nests of dysplastic epithelial cells that are either highly atypical or individually keratinized.

Diagnosis of MICA can be made only by histopathological examination. The criteria for diagnosis include desmoplastic reaction of the stroma, well matured squamous cells, with loss of polarity and necrosis within the lumen. Common age at presentation falls between 35 – 46 yrs. It amounts to about 20% of all cancers of the cervix.

Important parameters in microinvasive carcinoma:

Tumor type :

Certain subsets of squamous carcinoma, such as papillary squamous carcinomas, may not be as easily evaluated without complete excision.

Confluence of Growth Pattern :

Confluence has been defined as anastomosing tongues of epithelium with pushing borders or a lesion front of greater than 1 mm.

Capillary-Lymphatic Space Invasion:

The majority of cases with CLSI do not present with histologically positive lymph nodes, but CLSI is associated more commonly with an adverse outcome.

INVASIVE SQUAMOUS CELL CARCINOMA :

Carcinoma cervix is more common in women in their fourth or fifth decade though cases have been reported in all age groups above fifteen. The age group in which malignancy prevails is higher by nearly twenty years when compared to that of high grade intra epithelial neoplasia. However more of cases reported today are less than 30 years of age.

The clinical presentation of patients with invasive carcinoma of the cervix depends on the size and stage of the lesion .During the earlier periods, the patients presented with bulky masses since they had a very late diagnosis. Nearly all of them presented with clinically visible tumors and with complaints of profuse

vaginal bleeding. The other manifestations include spotting per vaginum, serosanguinous discharge, and frank bleeding .

The following features aid in the diagnosis of invasion:

- a desmoplastic response in the adjacent stroma.
- focal conspicuous maturation of the neoplastic epithelium with prominent nucleoli.
- blurring of the epithelial-stromal interface
- loss of polarity of the nuclei at the epithelial-stromal border with absence of the palisaded pattern characteristic of CIN
- scalloping of the margins at the epithelial-stromal interface
- the appearance of pseudocrypt involvement
- the apparent “folding or duplication” of the neoplastic epithelium

Duplication of epithelium refers to the presence of vascular structures within a sheet of neoplastic epithelial cells, producing an image of incompletely formed papillae

Cervical carcinomas metastasise locally into the cervical stroma, the paracervical and parametrial tissues, the body of the uterus, the vagina, the pelvic nodes and late in the course of the disease to the bladder and rectum.

Microscopically, overt invasive squamous cell carcinomas usually display considerable morphologic heterogeneity in growth pattern, cell type, and degree of cellular differentiation. However many are characterized by sheets and nests of

squamous cells invading into the stroma which shows a desmoplastic change. The tumor cells can also occur in single or in large masses entirely replacing the stroma.

The individual cells are polygonal with abundant cytoplasm and pleomorphic nuclei with coarse chromatin and prominent nucleoli. The mitotic figures are usually increased and the stroma may show infiltration by inflammatory cells.

Invasive squamous cell carcinomas are divided into two groups,

1. keratinizing
2. nonkeratinizing

Keratinizing carcinomas are characterized by the presence of well differentiated squamous epithelial cells that vary in size and configuration, arranged in nests or cords. The keratinizing carcinomas are defined by the presence of keratin pearls within the epithelium and the presence of a single keratin pearl is sufficient to classify as a keratinizing carcinoma. Keratin pearls are clusters of squamous cells arranged in a concentric nest, that have undergone keratinization .

Nonkeratinizing squamous cell carcinoma is characterized by nests of neoplastic squamous cells that do not form keratin pearls but frequently undergo individual cell keratinization with numerous mitotic figures. The cells have relatively indistinct cell borders and round to oval nuclei with coarsely clumped chromatin .

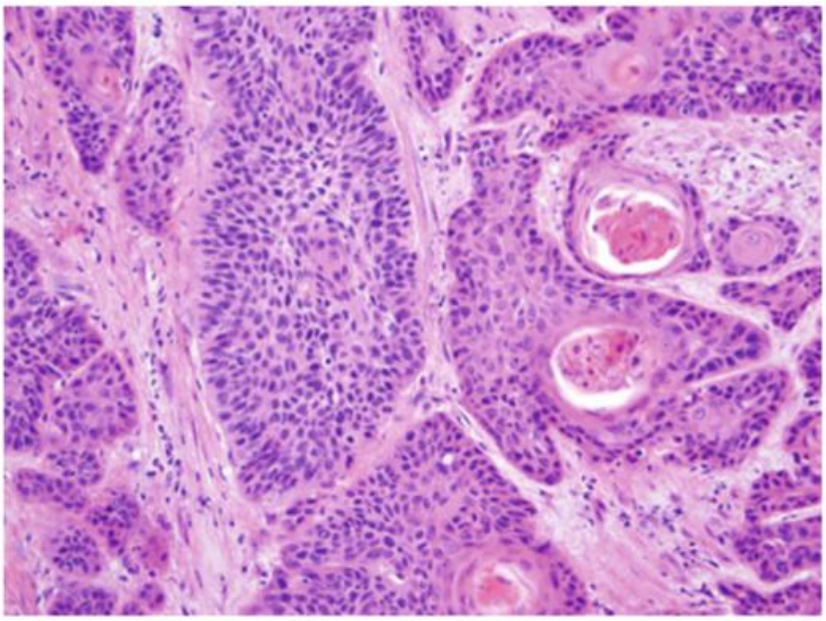

Fig.5. Microscopic appearance of well differentiated squamous cell carcinoma with keratin pearls

MICROSCOPIC GRADING OF SQUAMOUS CELL CARCINOMA:

Squamous cell carcinomas are graded histologically as follows:

1. Grade I -Well differentiated
2. Grade II -Moderately differentiated
3. Grade III - Poorly differentiated

Of the three, the most commonly occurring tumor belongs to grade II, moderately differentiated squamous cell carcinoma. Next frequent tumor would be grade III(poorly differentiated), followed by grade I (well differentiated) .

Well-differentiated squamous cell carcinoma:

This carcinoma is characterized by the presence of keratin pearls and individual cell keratinisation with prominent intercellular bridges. The tightly

packed cells have irregular , hyperchromatic nuclei with mitosis seen at the advancing margin of the tumor and the stroma showing inflammatory reaction with occasional giant cells of foreign body type as a reaction to the keratin.

Moderately differentiated squamous cell carcinoma:

The grade II tumor cells are large with indistinct cell margin and intercellular bridge with increased nuclear cytoplasmic ratio and irregular nuclei showing moderate degree of pleomorphism. Keratin pearls are absent. Tumor cells may show individual cell keratinization with increase in mitosis.

Poorly differentiated squamous cell carcinoma :

The tumor cells show high degree of pleomorphism with very scant cytoplasm and oval, hyperchromatic nuclei. Occasional giant cells with bizarre nuclei can be seen and there is total absence of keratinisation with heavily increased mitotic rate and focal areas of necrosis. Rarely the tumor cells can be spindle shaped.

VARIANTS OF SQUAMOUS CELL CARCINOMA :

Verrucous carcinoma

It is a rare variant, which is considered a highly differentiated type of squamous cell carcinoma .On gross it appears to be polypoid in nature, and has an extremely well-differentiated cytologic appearance. Some cases have been found to extend into the endometrial cavity.[32]

Spindle cell carcinoma (sarcomatoid carcinoma; squamous cell carcinoma with sarcoma-like stroma; carcinosarcoma)

The tumors frequently contain both squamous carcinoma and spindle cell components, either separate or subtly blending. They may contain osteoclast like giant cells, and there is often evidence of HPV infection.[33,34] The epithelial component of this tumor, when present, is usually of squamous type.

Basaloid (squamous cell) carcinoma

Basaloid carcinoma is characterized by prominent peripheral palisading, an infiltrative growth pattern, and minimal stromal reaction.[35] This tumor is aggressive and the differential diagnosis for this are the adenoid cystic carcinomas and adenoid basal carcinomas.

Lymphoepithelioma-like carcinoma

Lymphoepithelial-like carcinomas, which consist of poorly defined aggregates of nonkeratinized tumors cells, often with indistinct cytoplasmic borders, intermixed with abundant lymphoid cells, are similar to their morphologic counterparts in the pharynx.[36]

Transitional cell (urothelial) carcinoma :

This lesion has an appearance similar to the tumor located in the bladder or ovary . It needs to be distinguished from inverted transitional cell papilloma and papillary squamous cell carcinoma.[37]

Important parameters in squamous cell carcinoma:

Reporting Squamous Carcinomas Histologic reporting should include the following:

- Grade (well, moderately, or poorly differentiated)
- Cell type
- Depth of invasion or thickness
- Extent of tumor: the extent of invasion into extracervical tissues and metastases to both pelvic and extrapelvic organs should be recorded
- Invasion into the vessels and lymphatics
- The total number of nodes removed with how many being involved by metastases must be reported
- If the clearance given is adequate by careful examination of the resection margins.

ADENOCARCINOMA:

Primary adenocarcinomas make up 5–15% of all carcinomas of the cervix. The occurrence of adenocarcinoma of the cervix is on the rise in relation to squamous carcinoma.[38] Not all adenocarcinomas are associated with HPV. There are several different subtypes of adenocarcinoma, possibly with different etiologies and natural histories. Since these carcinomas have a tendency to grow endophytically they are diagnosed only very late. Further the tumor has a worser prognosis compared to squamous cell carcinoma.

Incidence is on the rise in the general population, particularly in young women. An association has been found between the long-term use of oral contraceptives and the development of endocervical neoplasia in young patients. Young age and exposure to HPV are other risk factors. Unresolved, however, is the mechanism by which these events increase the risk of glandular neoplasia relative to squamous neoplasia. The tumor shows no characteristic distinguishing gross features.

ADENOCARCINOMA IN SITU :

Though it predominantly involves the endocervix, the transformation zone is never spared. It has continuation with the overlying epithelium and invades the stroma widely. It is also usually associated with intra epithelial lesions. Rarely AIS initially develops high in the canal or is multifocal.[39]

The neoplastic glands are lined by stratified columnar cells that are oriented vertical to the basal layer. The basally located nucleus is oval, and hyperchromatic with mild pleomorphism .

EARLY INVASIVE ADENOCARCINOMA:

Assessing early invasion in glandular lesions is some- times difficult because, unlike squamous lesions, preservation of AIS-like gland architecture may accompany invasion. Conversely, AIS itself may be quite complex. Thus, it is generally not advisable to diagnose early invasion from small biopsies. It is wise to have a larger one.

The subtypes include

- endocervical
- endometroid
- intestinal
- tubal
- stratified

It does not show desmoplastic reaction though inflammatory cells can be seen.

Hence the features to make a diagnosis include:

- Epithelial cell crowding- This occurs with stratification or pseudo stratification.
- Nuclear enlargement- Nuclei are variably enlarged and are oval, elongated, or irregular
- Prominent nuclear hyperchromasia with chromatin coarsening- Nucleoli are usually small or absent but may be prominent in some cases
- Mitotic figures - These are invariably present, often easily visualized at the luminal pole of the cell, but there may be as few as one or two per five high-power fields.
- Apoptotic bodies - One should require a definitive eosinophilic body containing sharply delineated dark fragments of chromatin so as not to confuse degenerated leukocytes with apoptotic bodies

- Conspicuous architectural alterations-These sometimes include papillary or cribriform intraglandular growth and may be quite florid, suggesting invasive carcinoma.

Adenocarcinoma in situ is associated with CIN in at least 50% of cases and is immunoreactive for carcinoembryonic antigen in 80% of cases.[40]

INVASIVE ADENO CARCINOMA:

There are three patterns of invasion that characterise adenocarcinomas, and all of them may be seen in early neoplasms. They are

i. Infiltrative :

Its earliest manifestation is characterized by small protrusions into the stroma that appear to bud from AIS glands, often with an accompanying inflammatory response. The cells of the buds and the early invasive glands are often larger than the AIS cells, with large nuclei, sometimes prominent nucleoli, and an expanded eosinophilic cytoplasm. The architectural pattern of adjacent benign glands provides a useful comparison.

ii. Expansile :

Here, well-circumscribed, AIS-like glands expand into the stroma as a compact unit rather than as separate infiltrative glands. Often there is no stromal reaction. This pattern, when it extends deeply enough, is recognized intuitively as invasive carcinoma.

iii. Exophytic :

This invasive pattern refers to surface polypoid or papillary growth that is more pronounced than the small papillary excrescences that are sometimes present with AIS (Fig. 14-41). Invasion of the underlying stroma may or may not be present.

Microscopically, the most common pattern is that of a well-differentiated glandular pattern with mucin secretion, some of which can leak into the stroma.

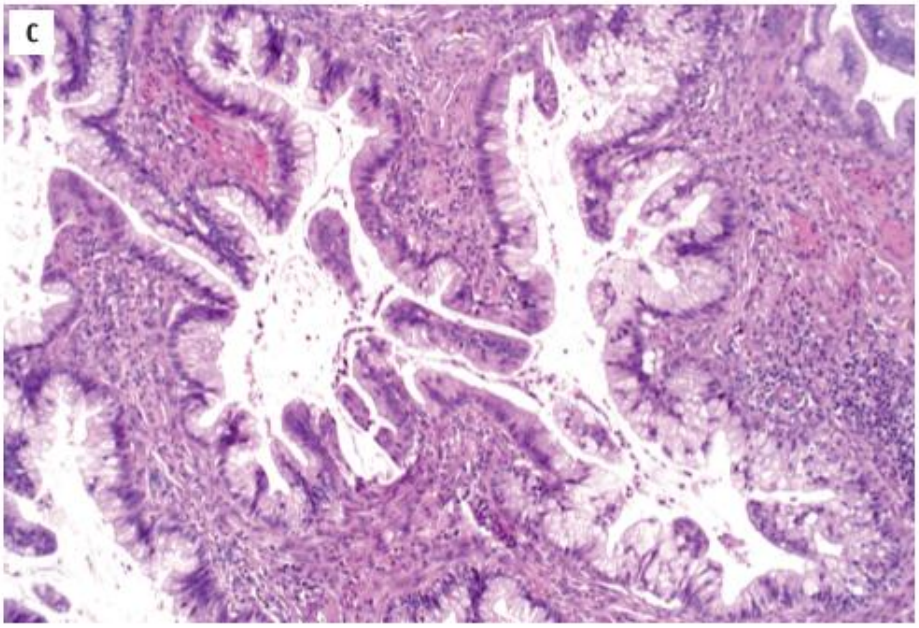

Fig. 6. Histologic appearance of well differentiated adeno carcinoma

VARIANTS OF ADENOCARCINOMA:

The variants include:

- Endometriod
- Serous(papillary)
- Clear cell

- Adenoid cystic
- Mucinous
- Villo glandular
- Adenoid basal
- Mesonephric adeno carcinoma

NEURO ENDOCRINE CARCINOMA OF CERVIX:

Small cell neuroendocrine carcinoma is morphologically similar to the classic oat cell carcinoma of the lung which has many of the following features:

- uniform cell population
- hyperchromatic nuclei
- a high nuclear–cytoplasmic ratio
- tumor cells arranged in irregular aggregates, often with little cohesion and
- occasional rosettes or poorly defined acini.

In addition, the nuclei contain coarse to opaque chromatin and, because there is little cytoplasm, they often appear to "mold" with adjacent nuclei. These tumors may also extensively infiltrate the underlying cervical stroma.

PROGNOSTIC PARAMETERS IN CARCINOMA CERVIX:

The prognostic parameters include the following:

- clinical staging
- size of the tumor

- depth of invasion
- age of the patient
- lymph node metastases
- parametrial extension

Among them, the most significant one is the clinical staging of the tumor.

Staging of the disease:

The clinical staging of the disease at the time of diagnosis is an important prognostic factor and it determines the survival of the patient. Generally stage I has better survival rate. In stage I disease, there is difference in survival rate between IA and IB. Patients belonging to stage IA has 5-yr survival rate of 95% which gets reduced to about 80% - 90% in patients with stage IB. (Annexure-II)

Survival rate gets reduced to about 75% in cases with stage II and lower to 50% for stage III. Stage IV patients usually presents with local metastasis to adjacent kidney, bladder, urethra, at the time of diagnosis. So the survival of these patients is generally reduced and they die of complication due to local metastasis.

Histological type :

Poorly differentiated carcinoma and small cell carcinoma have bad prognosis than other types. The 5 year survival rate of adenocarcinoma is 10% lower than that of squamous cell carcinoma.

Size of tumor:

Generally, smaller the size of tumor better the prognosis. The 5-yr survival rate of tumors of size less than 3 cm is about 86%. Tumors of size 3-5 cm has 5 yr survival rate of about 76% and for tumors of size larger than 5 cm ,the 5-year survival rates is around 61.5%.

Depth of stromal invasion :

It is the distance to which tumor has extended into the stroma and measured from the basal layer of the epithelium to the deepest point of tumor extension. With increase in the depth of invasion, there is a high chance for the parametrial extension, lymph node metastasis and recurrence. The stroma free of tumor serves as a barrier to the spread of tumor.[41]

Lymphovascular invasion:

The lymphovascular invasion is defined as the presence of tumor mass within any space lined by endothelial cells and adherence of tumor cells to the endothelial cells.[42] When the depth of invasion is more, there is increased chance for the occurrence of lymphovascular invasion. LVSI is related with decreased survival rate and increased risk of recurrence.

Parametrial involvement :

The parametrium is infiltrated by tumor cells either by contiguous spread and to lesser extent from lymphatic spread. Survival rate of stage I and II cancers is 90% without parametrial involvement, which gets reduced to 77% with

parametrial involvement.[42] Involvement of parametrium with tumor shows high incidence of lymphnode metastasis, blood vessel invasion, tumor recurrence and increased mortality.

Nodal status :

The metastasis of lymph node depends on several factors like clinical staging, size of tumor, distance of stromal invasion, lymphovascular invasion and grade of tumor.[42] As the tumor advances to stage III and IV, there is increased chance of lymph node metastases.

The cervical cancer spreads to obturator, paracervical and external iliac nodes. Pelvic node metastasis carries worse prognosis. Rarely there is involvement of para-aortic lymph nodes and if present it indicates dissemination of cancer. In the presence of pelvic lymph node metastasis, the 5 yr survival rate is observed to be reduced.

Mitotic figures in dysplastic and malignant lesions of the cervix:

The stratified squamous epithelium of the ectocervix is so programmed that it undergoes an orderly maturation from the basal to the most superficial layer. The changes that can be seen from bottom to top include condensation of nuclei with increase in chromatin density and increase in the amount of cytoplasm due to the accumulation of glycogen.

The parabasal cells in the epithelium serve the function of active proliferation and hence the mitotic activity is predominantly confined to the lower

two third of the epithelium. These mitotic figures can be studied in various aspects such as their distribution, normal or abnormal and density to understand the proliferative potential of the lesion. Also these elements can be demonstrated by immuno histochemistry. Their major application in pathology lies in distinguishing low grade from high grade intra epithelial lesions and to differentiate high grade dysplasia from other mimickers like atrophy and immature metaplasia.

The occurrence of increased numbers of mitotic figures in dysplasias and carcinoma in situ have been mentioned by several. But there are no reports dealing with their exact frequencies.

IMMUNOHISTOTECHNIQUES :

Immunohistochemistry is one of the ancillary techniques that helps in identifying the tissue of origin on the basis of antigen antibody reaction. The antibody that is bound can be identified by labelling it either directly or by using another antibody i.e. secondary antibody. These antibodies have significance in that they serve as both prognostic and predictive markers.

The success of this technique lies in the accurate localisation of the antibody that has combined with the antigen and its precise visual identification. This is mainly done by amplification that multiplies the signal so that it is easy to visualise. At the same time there must not be any interference from the background staining. Immunohistochemistry helps the pathologists to fit the tumor, that has been classified as undifferentiated in histology , into a specific histogenetic origin.

Fixation and Other Pre-Analytical Factors :

Autolysis is the process of self destruction by enzymes within the cellular organelles. The primary aim of fixation is to retard this process so that the internal architecture is preserved.

There are many fixative recipes, but most of these can be grouped into three main categories as per the constituents as those that contain formalin, alcohol or both.

Tissue Processing:

It is the process during which the sections are passed through increasing grades of alcohol from 70 to 100% to remove water from the section .This is followed by wax impregnation which removes the alcohol. Waxes with low melting temperature (45 °C) give better staining results for IHC. The sections are then embedded in paraffin.

Section Preparation:

Sections of 3-5 micron thickness are preferred. They are preferably cut using a fresh blade. The sections are thin so that they are not washed out during antigen retrival Further thick sections hinder with staining due to multiple layers.

Coated slides are available to aid in firm attachment of the section to the slide. They are positively charged so that they firmly attach to the tissue proteins that are negatively charged. Sections must be firmly adhered to the glass to prevent lifting during staining or bubble formation, which may trap staining reagents.

Antigen Retrieval:

Antigen retrieval by heating is an imporrtant step in IHC. When the sections are heated to a high temperature, the tissue proteins are subjected to hydrolysis which breaks the cross links thus exposing them for the antibody to bind. For many antigens, almost any kind of heating treatment, including microwave oven, water bath, pressure cooker, or autoclave may generate similar results, if adjusted appropriately for time.

The pH value of the AR solution is another factor that significantly influences the results. A higher pH AR solution, such as Tris-HCl or sodium acetate buffer at pH 8.0-9.0, may be suitable for most antigens .

Antibody:

Antibodies belong to the class of serum proteins known as immunoglobulins.

They are found in blood and tissue fluids, as well as many secretions. The basic unit of each antibody is a monomer. An antibody can be monomeric, dimeric, trimeric, tetrameric, or pentameric.

The antibodies used can be:

1. Polyclonal
2. Monoclonal

Production of polyclonal antibodies:

Polyclonal antibodies are produced by immunizing an animal with a purified specific molecule (immunogen) which has the antigen of interest. The animal will mount a humoral response to the immunogen and the antibodies so produced will be a combination of many clones of activated plasma cells. The cells of each clone vary particularly in their specifity to the different epitopes that are expressed by the immunogen.

Synthesis of monoclonal antibodies:

The principle behind the production of monoclonal antibodies uses a combination of two properties- one is the ability to produce a specific antibody by an activated B lymphocyte or plasma cell and the other being the immortal property of malignant myeloma cells. These cells can be cultured in appropriate medium to multiply in huge numbers. This is referred to as the hybridoma technique.

Antibody-antigen binding:

The antibody combines with the epitope of the antigen by means of amino acid side-chains which are complementary to each other. The combination of an antibody with an antigen requires high specificity. The interaction between the antibody and the antigen can be either alone or a combination of hydrogen bonds, electrostatic interactions, and van der Waals' forces.

Enzyme labels:

Enzymes as labels have a wide application in immunohistochemistry such as in blocking unwanted background staining and as chromogen that gives a coloured product so that it can be easily visualised by light microscope. The enzyme that is usually used is the Horseradish peroxidase in conjunction with the most favoured chromogen, i.e. 3,3α-diamino benzedene tetrahydrochloride (DAB), This is preferred because it gives a crisp, stable, dark brown end-product.

If the coloured reaction products are miscible with alcohol,mounting should be done in an aqueous medium.

Blocking background staining:

The major causes of background staining in immunohistochemistry are the hydrophobic and ionic interactions and endogenous enzyme activity. Background staining may be specific or non-specific due to the apparent affinity of certain tissue components. Non-specific uptake of antigen, particularly the high affinity of collagen and reticulin for immunoglobulins, can cause high levels of background staining.

One of the ways to minimise this staining is to add an innocuous protein solution to the section before applying the primary antibody. The added protein should saturate and neutralize the charged sites, thus enabling the primary antibody to bind to the antigenic site only.

The blocking of endogenous enzymatic activity must be carried out before the addition of enzyme-labelled secondary reagent; otherwise, the enzyme label is also inactivated by the blocking procedure, resulting in a false- negative result.

As already mentioned the antibody molecules that take part in this reaction cannot be seen with the light microscope or even with the electron microscope unless some chemical in the name of labels or flags is used for their visualisation. Hence the detection systems tag labels or flags to primary or secondary antibodies to visualize the target antibody antigen localization.

Washes:

To prevent the formation of antigen-antibody complexes that will precipitate onto the sections and give rise to problems with interpretation and back- ground staining, it is necessary to remove the unbound antibody before incubation in the next layer. This is achieved by washing the sections in Tris-buffered saline (TBS). This solution may be made up in bulk for convenience. For routine work, a few brief washes, with TBS, will usually suffice.

PRECAUTIONS

1. The glasswares used should be dry and clean.
2. All the buffers used should be prepared fresh and the pH should be adjusted according to the preferred pH.
3. The staining procedures are never allowed to dry so they are performed under a humidity chamber.

4. DAB chromogen should be handled and disposed carefully as it is a carcinogen.
5. Primary and secondary antibody, DAB chromogen, peroxidase block, and amplifier must be stored at 4-6ºC
6. While performing IHC every batch should have a positive control slide.

Ki-67 AND p16 AS BIOMARKERS IN CARCINOMA CERVIX:

Ki-67:

Ki-67 is a nuclear protein that is expressed by multiplying cells in all the phases of cell cycle(G1, S, G2, M) except G0.It can predict the possibility of development a tumor and so is called a proliferation marker. The level of Ki-67 expression is used to determine the cell proliferation status[43,44]. Its expression is usually seen in a normal mucosa and the lowermost layer of the epithelium. However when it shows positivity in the rest of the layers it implies disordered maturation.

Although Ki-67 has been used as a diagnostic adjunct for the classification of cervical tissue specimens[45,46], the expression of Ki-67 alone does not discriminate HPV-mediated dysplasia versus benign proliferating cells in benign reactive processes.

P16:

p16INK4a, a cyclin-dependent kinase is a tumor-suppressor protein. Its action of inhibition is by blocking cdk4- and cdk6-mediated phosphorylation of

Rb gene which in turn down regulates E2F-dependent transcription. Since this transcription has an essential role in cell-cycle progression, there is no further proliferation[47]. This functional inhibition of pRb by HPV E7 leads to increased expression of p16INK4a followed by its accumulation within the cells.

1. p16INK4a is thus a surrogate marker of HPV E7-mediated pRb catabolism, providing evidence of transformation of the cervical mucosa[48,49]. The reason for using p16INK4a in classifying HPV-related lesions include: [50]

2. Since there must be a continuous expression of E7 for the maintenance of the malignant phenotype, the expression of p16INK4a has a direct correlation with the malignant potential of HPV oncogenic action

3. Since p16 positivity is independent of the specific HPV type, genotyping is not necessary

4. The expression of p16INK4a is a specific indication of HPV-E7 overexpression or other events that lead to silencing of Rb[51].

High grade intra epithelial neoplasia i.e. CIN-II ,CIN-III and squamous cell carcinoma of the cervix show diffuse staining of p16 .But in benign lesions of the cervix and low grade CIN-I which seem to be associated with low risk HPV, the expression is found to be very minimal or absent. The other conditions where focal and occasionally diffuse expression can be observed include benign

endocervical intercalated columnar cells, tuboendometrial metaplasia, and cervical endometriosis[52].

The expression of p16INK4a in these cells, however, denotes no premalignant potential. There can be focal staining seen in the lower 1/3rd of CIN1 lesions and in the upper third can show positivity in cases of squamous metaplasia. Only a diffuse staining of p16INK4a in the lower third of the epithelium, is considered specific for CIN1 lesions. Diffuse expression of p16 in glandular epithelial cells usually implies endocervical glandular neoplasia, Adeno carcinoma Insitu, or invasive adenocarcinoma.

Many studies however take into consideration a dual staining that combines p16INK4a and Mib-1 and a positive scoring is done on the basis of individual cells which does not take cell morphology as a criteria.[53,54]

OTHER PROGNOSTIC BIO MARKERS:

LRIG:

LRIG, leucine-rich repeats, and immuno- globulin-like domains 1 (LRIG1) is a transmembrane protein, whose expression is considered a good prognosis particularly in early stages of cervical cancer.[58] It acts by inhibiting growth factor signaling by increasing ubiquitylation followed by degradation of epidermal growth factor receptor (EGFR) .It can also act by inhibiting the cellular oncogenes Met and Ret.

COX-2 :

Several studies on High cyclooxygenase 2 (COX-2) reveal that its expression has a poor prognosis[55]. It is an essential enzyme in the conversion of arachidonic acid to prostaglandins and when it is expressed in large amounts it amplifies the metastatic potential and angiogenesis, and at the same time decreases host defense and apoptosis.

EGFR2/HER2:

The rapid progress of the tumor and resistance to therapy in malignancy of the cervix can be assessed by studying the expression of EGFR and human EGFR2 (HER2) .Its expression is associated with poor prognosis[56].

D2-40 :

D2-40 is a marker for endothelium that lines the lymphatic vessels. By evaluating the number of vessels that show positivity peritumoral lymphatic vessel density can be studied. It has a significant correlation with advanced tumor stage, lymph node metastases, and poor survival particularly in squamous cell carcinomas.[57]

VEGF:

Overexpression of VEGF has been associated with tumor progression and poor prognosis in several tumors, including cervical cancer. Intratumoral protein levels of VEGF are increased in cervical cancer compared to normal cervical tissue and higher VEGF levels correlate with higher stage and increased risk of lymph

nodes metastasis . It has also been demonstrated that higher VEGF expression and increased tumor vascularisation are independent predictors of shorter disease-free interval and overall poor survival .

P53 and RAS:

P53 and RAS mutations are not commonly expressed in cervical malignancies and in recent studies they do not seem to be associated with tumor stage, tumor grade, or survival. However amplification and overexpression of cellular c-myc zncogene has been proved to be associated with HPV16. Overexpression of c-myc has also been associated with worse prognosis in some studies .

CYCLIN E:

Normal epithelium does not express Cyclin E, a nuclear protein that gets up-regulated by HPV 16 E7 directly linked to viral replication. It can be used to differentiate between low and high grade lesions when expressed in the nuclei.

CD 4 positive lymphocytes :

Infiltration of CD4 positive lymphocytes of tumor cells indicates better survival. Its expression is associated with good prognosis.

MATERIALS AND METHODS

STUDY LOCATION:

The study was undertaken in the Department of Pathology, Tirunelveli, Medical College.

STUDY PERIOD:

The study was conducted from the years 2013 to 2015.

SAMPLES:

A total of 50 cases including cervical intra epithelial neoplasia and carcinoma cervix.

INCLUSION CRITERIA:

1. Cervical biopsies diagnosed cervical intra epithelial neoplasia (I, II, III) and carcinoma cervix.
2. Hysterectomy specimens diagnosed cervical intra epithelial neoplasia (I, II, III) and carcinoma cervix.

EXCLUSION CRITERIA:

1. Cervical biopsies reported as inflammatory conditions
2. Cervical biopsies diagnosed mesenchymal lesion.
3. Hysterectomy specimens for causes other than carcinoma cervix.

METHODOLOGY:

DATA COLLECTION:

The data including patients age, clinical staging, and other clinical data were obtained from the pathology records.

PROCESSING OF SPECIMEN:

Cervical biopsy specimens and hysterectomy specimens received were fixed in 10% formalin and processed routinely. Biopsy specimens were presented in to and in hysterectomy specimen, cervix was carefully sectioned to include endocervix and ectocervix.

STAINING TECHNIQUE:

Sections of 4-5µ thickness were cut and stained with Haematoxylin & Eosin. The slides were studied under light microscopy and the data recorded.

IMMUNOHISTOCHEMICAL EVALUATION:

Immunohistochemistry was performed on 3-4m-thick sections taken on poly-L-lysine-coated slides. Antigen retrieval was performed by heating the sections in Tris-EDTA buffer at pH 6.0 using pressure cooker. Mouse Monoclonal antibody was used to bind with the primary antigen and is detected by adding secondary antibody conjugated with horse radish peroxidase – polymer and diaminobenzidine substrate. In this study, Ki-67 and p16 antigens of Pathnsitu laboratory products is used.

PROCESSING FOR IMMUNOHISTOCHEMISTRY :

- 3µm thickness sections were cut using fresh blade in microtome from the chosen paraffin blocks.
- The floated sections were taken in adhesive slides coated with poly-L-lysine.
- The slides were incubated overnight at 60 c.
- The slides were subjected to 2 changes of xylene , 5 minutes each for deparaffinization.
- They were then transferred to absolute alcohol for 5 minutes followed by 80% and 70% alcohol for 5 minutes to rehydrate the tissue sections.
- Tissue sections were then washed in distilled water.
- Antigen retrieval was done using pressure cooker in Tris-EDTA buffer.
- The sections were then cooled to room temperature and the slides washed with distilled water
- Endogenous peroxidase activity was removed by incubating the tissue sections with enough drops of 3% peroxide block in a humid chamber for 5minutes.The sections were then washed in TRIS wash buffer.
- Primary antibody was then added to cover the tissue sections and incubated for 30 minutes.
- The tissue sections were then washed in TRIS wash buffer followed by

- amplifier for 15 minutes to enhance the activity of primary antibody which were then washed in TRIS wash buffer.
- Secondary antibody was then added and incubated for 20 minutes followed by washing with TRIS wash buffer.
- DAB chromogen (1ml DAB buffer +1 drop DAB chromogen) was then added and incubated for 4 minutes and then washed with 2 changes of distilled water.
- Counterstaining was done with haematoxylin for 30 seconds and washed in running tap water.

This was followed by dehydration, clearing and mounting.

SCORING OF Mib-1:

Grading of Ki-67 expression:

For Ki-67, immunopositivity was considered when there was strong nuclear staining. Because basal staining is a normal finding, the slides were first assessed for basal staining (lower one-third versus suprabasal). Staining in the upper two-third of the epithelium was considered positive.

The sections stained for Ki-67 proliferation (revealed as nuclear staining) were evaluated using scores from 1 to 3[59] :

Table 2 : Grading of Ki-67 expression

RATE OF POSITIVITY	DEGREE OF PROLIFERATION	GRADING
>50%	High Proliferation	+++(3)
30-50%	Moderate Proliferation	++(2)
10-30%	Low Proliferation	+(1)

Calculation of MIB-1 Labelling Index[60]

MIB-1 labelling index (LI) was calculated by the number of positive cells per 100 cervical epithelial cells in different areas under X400 magnification and the mean was calculated. Positive nuclei were expressed as the percentage of total nuclei counted.

MIB-1 labelling index was calculated as follows:

$$\frac{\text{No of cells showing positive staining}}{\text{Total No of cells}} \times 100$$

P16 scoring:

For p16, immunopositivity was considered when there was diffuse, strong, nuclear and/or cytoplasmic staining. Heterogeneous or focal moderate nuclear staining was also considered positive. Weak cytoplasmic staining was considered

negative. Grading was then performed for each case by the number of positive cells in different epithelial clusters as follows[61]:

Table 3 : Scoring of p16 expression

GRADE	POSITIVITY
0	--
1	1-10%
2	10-50%
3	>50%

Evaluation of mitotic figures:

In each case, the entire evaluable cervical epithelium was surveyed in a consistent manner by moving the microscopic field from the superficial to deep layers. Therefore, the total surface area of cervical epithelium evaluated was dependent on the number of sections initially obtained. Approximately 1000 cells were assessed under high power magnification for each specimen and the mitotic count is calculated as a percent for 100 cells.

OBSERVATION AND RESULTS

Table 4 : Distribution of cases

Diagnosis	No. of cases
CIN-I	14
CIN-II	8
CIN-III	6
Carcinoma	22

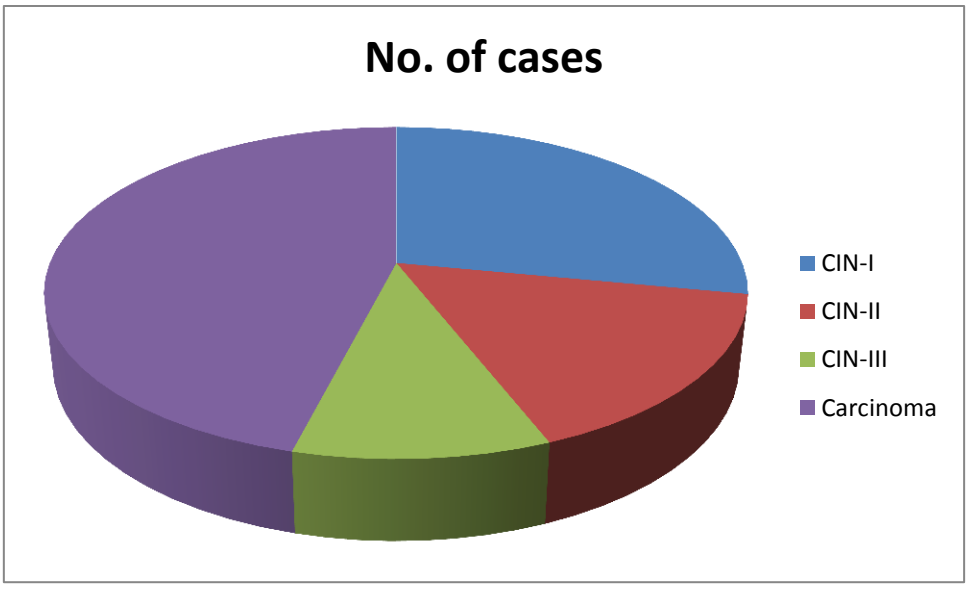

Chart:1 Distribution of study cases

Table 5 : Distribution of cases in carcinoma cervix

Histological type	No.of cases
Large cell non keratinising squamous cell carcinoma	**13**
Large cell keratinising squamous cell carcinoma	**8**
Adenocarcinoma	**1**

Table 6 : Comparison of mean of mitotic count

	Mean mitotic count
CIN-I	0.85
CIN-II	1.3
CIN-III	1.98
LCNK	2.6
LCK	2.5
Adeno	0.9

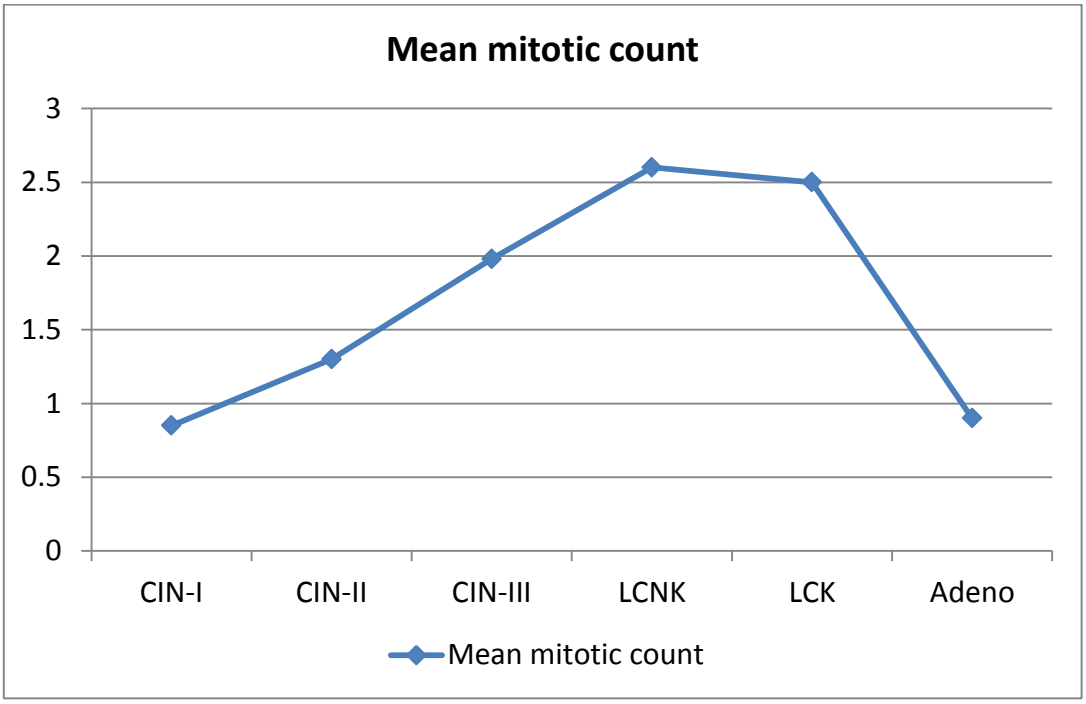

Chart : 2 Comparison of mean of mitotic count

From the above table and the chart we infer that as the degree of dysplasia increases, the mean mitotic count also increases. Hence the mitotic count can be used to study the proliferative potential of the lesion. However the mitotic count in adenocarcinoma is less when compared to that in squamous cell carcinoma which may be due to its low proliferative potential.

Table 7 : Correlation between Ki-67 and p16 expression in study cases

P16			
Ki-67		**Positive**	**Negative**
	Positive	28	11
	Negative	3	8

p value=0.013 (Fisher's exact test)

The above table shows a definite association between the expression of Ki-67 and p16 in cervical lesions (both benign and malignant) since 28 cases out of the 50 cases showed positivity for both the markers. Only 8 turned out to be negative for both. P value is <0.05 and hence it is also statistically significant.

Table 8 : Comparison of Ki-67 and p16 positivity in study cases

		Ki-67		P16	
	Total	Positive	%	Positive	%
CIN-I	14	9	64.2	3	21.42
CIN-II	8	5	62	5	62
CIN-III	6	6	100	5	83
LCK	8	8	100	8	100
LCNK	13	12	92.3	9	69.2
Adeno	1	0	0	0	0

P value=.0917(Pearson Chi-square test)

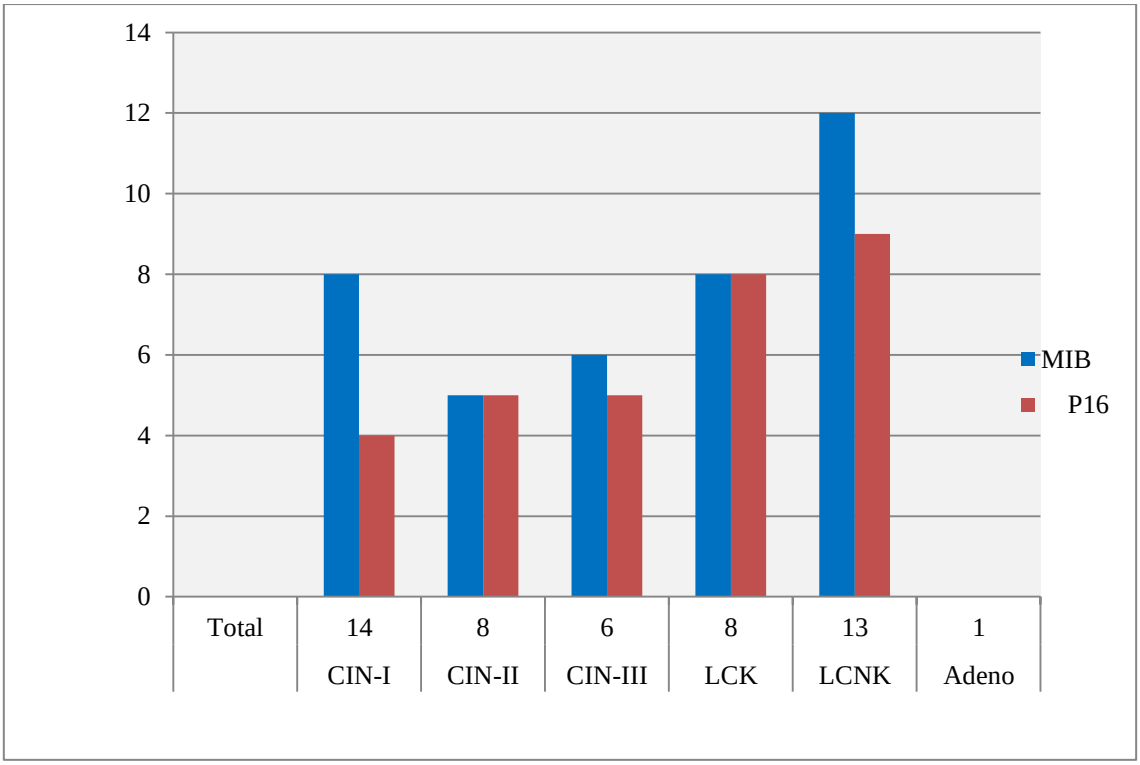

Chart : 3 Comparison of Ki-67 and p16 positive cases

The above table and the graph show that there is a expression of both Ki-67 and p16 together in both CIN and carcinoma cervix. Further as the severity of the dysplasia increases, the positivity rate also increases.

P16 showed only 21% positivity in CIN-I lesions whereas in high grade dysplasias the positivity was above 60%. This may be due to the association of HPV with high grade dysplastic lesions and malignancies of the cervix. However large cell non keratinising carcinomas showed only 70% positivity which emphasises the need to study if HPV is associated in these malignancies.

Ki-67 showed around 60% positivity even in CIN-I lesions though the grade was low. This rate keeps increasing then onwards.

Table 9 : Comparison of Ki-67 and p16 grading in CIN-I

S.No	Ki-67	P16
1	2+	0
2	2+	0
3	2+	1+
4	0	0
5	3+	0
6	1+	2+
7	2+	0
8	1+	0
9	2+	0
10	0	1+
11	0	0
12	0	0
13	1+	0
14	0	0

The above table shows variable expression of Ki-67 in CIN-I. Of the 13 cases,4 turned out negative whereas 1 showed 3+ positivity. The remaining showed 1+ and 2+positivity. This means that not all CIN-I cases have low malignant potential and a careful follow up is necessary. However the overall expression of p16 in CIN-I turned out to be low. Only 3 of them were positive. This may be due to the low association of HPV with these lesions.

Table 10 : Comparison of Ki-67 and p16 grading in CIN-II

S.No	Ki-67	P16
1	3+	2+
2	0	0
3	0	1+
4	0	0
5	1+	0
6	1+	2+
7	1+	3+
8	1+	1+

Nearly 50% of cases showed expression of Ki-67 and p16. 50% of cases showed 1+ Ki-67 positivity.3 turned out negative and one showed 3+ positivity. There was higher degree of expression in p16 compared to Ki-67 with only 3 of them being negative.

Table 11 : Comparison of ki-67 and p16 expression in CIN-III

S.No	Ki-67	P16
1	1+	1+
2	3+	1+
3	2+	1+
4	3+	2+
5	3+	0
6	1+	2+

Nearly all cases showed positive expression of both ki-67 and p16.In ki-67v positive cases, >50% showed 3+ and the remaining showed 1+ / 2+ positivity. This means not all CIN-III lesions have a high proliferation index where conservative treatment may be thought of P16 expression was negative in only one case that showed 3+Ki-67 positivity. The other cases showed 1+/2+ positivity which may be due to the low viral load in these cases. Further proliferation need not always be associated with HPV.

Table 12 : Comparison of ki-67 and p16 expression in large cell non keratinising carcinoma

S.No	Ki-67	P16
1	3+	0
2	3+	1+
3	3+	0
4	3+	3+
5	0	0
6	3+	3+
7	2+	2+
8	3+	3+
9	3+	2+
10	2+	2+
11	3+	3+
12	3+	0
13	3+	1+

The positivity of ki-67 and p-16 expression is very high in large cell non keratinising carcinoma. Ki-67 shows 2+ and above positivity in all cases except for one which showed no expression.p16 also showed 2+ and above positivity in more than 50% of cases.4 of them showed no expression. One case was negative for both the markers which may imply its low proliferative potential.

Table 13 : Comparison of ki-67 and p16 expression in large cell keratinising carcinoma

S.No	Ki-67	P16
1	3+	2+
2	3+	3+
3	2+	2+
4	3+	2+
5	3+	1+
6	2+	3+
7	2+	2+
8	3+	3+

The above table shows that there is a high degree of both ki-67 and p16 expression in large cell keratinising carcinoma.i.e.2+/3+positivity.

Table 14 : Ki-67 and p16 expression in adeno carcinoma

S.No	Ki-67	P16
1	0	0

There is no expression of either ki-67and p16 in adeno carcinoma. However since only one case has been studied, its significance cannot be proved.

Table 15 : Comparison of expression of Ki-67 in the study cases

Ki-67	0	1	2	3
CIN-I	5	3	5	1
CIN-II	3	4	0	1
CIN-III	0	2	1	3
LCK	0	0	4	4
LCNK	1	0	2	10
Adeno	1	0	0	0

The table shows that the CIN-I lesions predominantly show either no or grade 1 expression. An exception is one of them showing grade III expression. The carcinomas show either grade 2 or 3 expression which may point towards their aggressiveness .Adenocarcinoma showed no expression.

Table 16 : Comparison of expression of p16 in the study cases

P16	0	1	2	3
CIN-I	10	2	2	0
CIN-II	3	2	2	1
CIN-III	1	2	2	1
LCK	0	1	3	4
LCNK	4	2	3	4
Adeno	1	0	0	0

P16 did not show expression in many of the CIN-I cases whereas CIN-II and III showed some positivity in all cases. The squamous cell carcinomas showed higher grades of positivity. Thus as the severity increases, the degree of positivity also increases.

Table 17 : Comparison of mean if Mib-1 labelling index

Diagnosis	Mib-1 LI
CIN-I	13
CIN-II	15.75
CIN-III	36.8
LCK	44.8
LCNK	45.4
Adeno	0

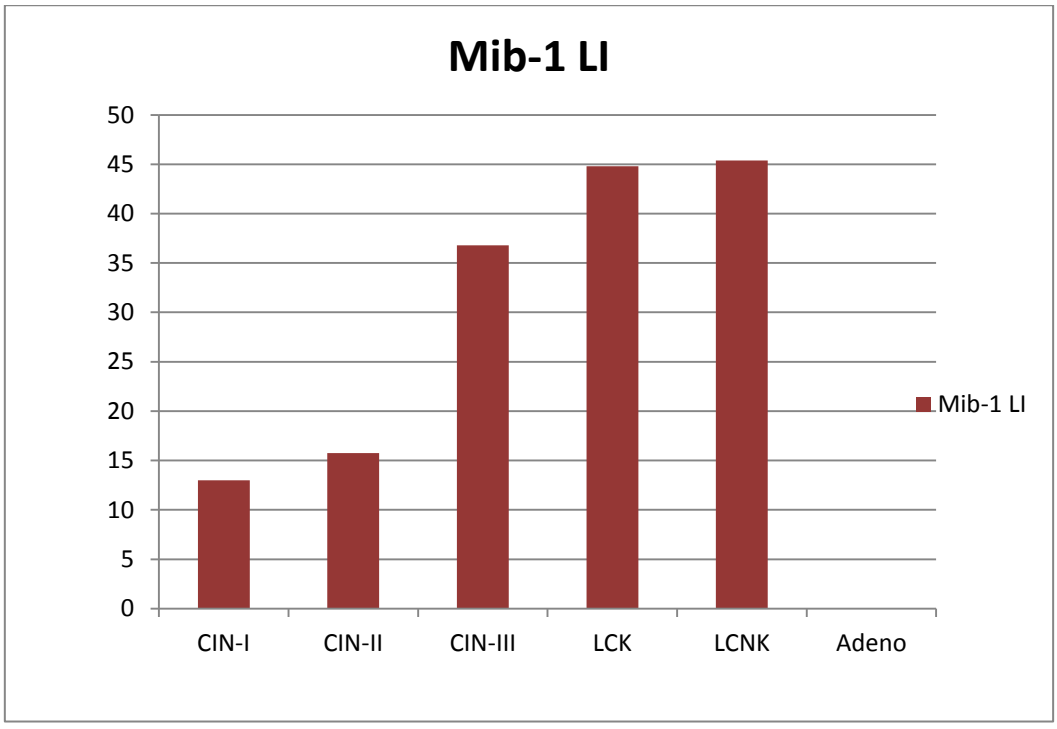

Chart : 4 Comparison of mean of Mib-1 labelling index

The above graph shows as the lesion progresses through increasing grades of dysplasia, the Mib-1 labelling index also increases which implies that the malignant potential increases.

Table 18 : Comparison of mean of mitotic count with that of Mib-1LI

Diagnosis	Mean Mib-1 LI	Mean mitotic count
CIN-I	13	0.85
CIN-II	15.75	1.3
CIN-III	36.8	1.98
LCK	44.8	2.6
LCNK	45.4	2.5
Adeno	0	0.9

P value (Pearson chi square test) = 0.014

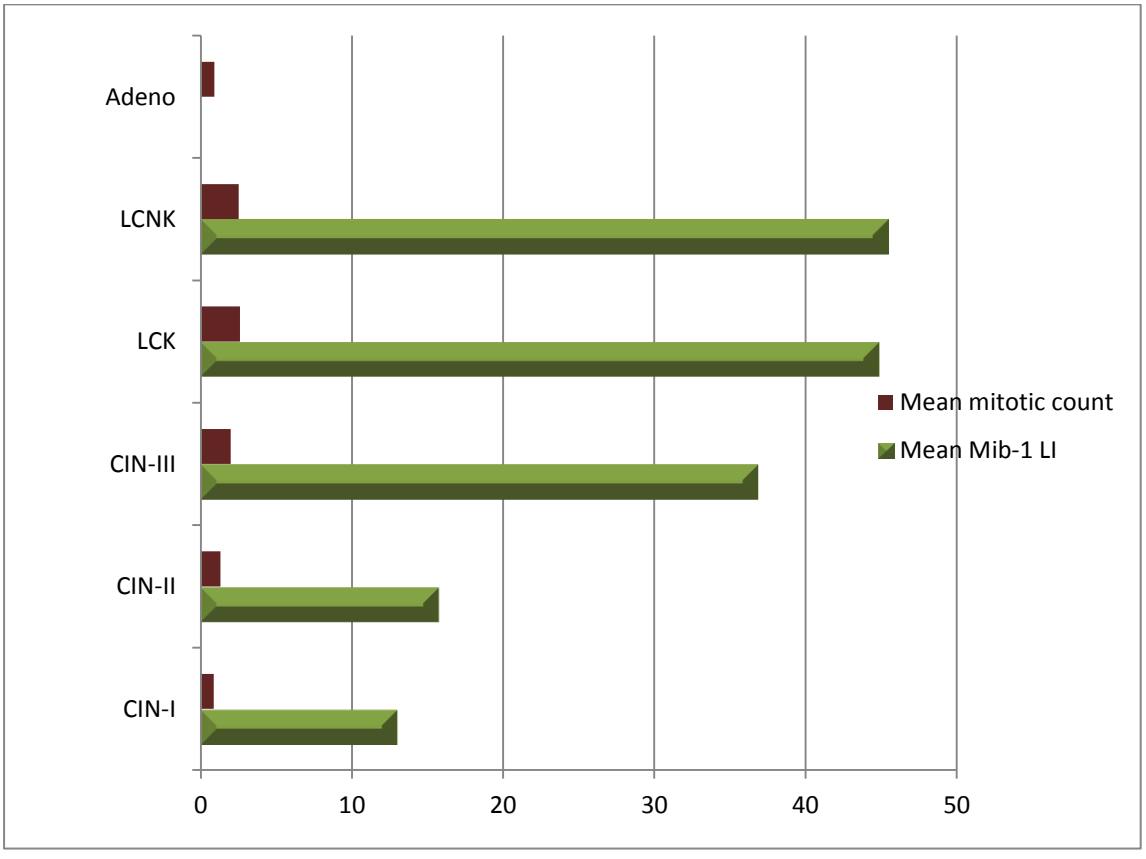

Chart : 5 Comparison of mean mitotic count with mean Mib-1 LI

From the above table, we infer that the mean of mitotic count and Mib-1 LI increase with increasing dysplasia. This association also has statistical significance (p value<0.05).

Table 19: Comparison of mitotic count with Ki-67 and p16 expression

		Pearson Correlation	P Value
Mitotic count	MIB	0.587	<0.0001
	P16	0.372	0.008

P value being significant shows an association between the mitotic count and the expression of Ki-67 and p16 individually.

Fig. 6. High power photomicrograph of a case of CIN-I showing 3+nuclear positivity of Ki-67

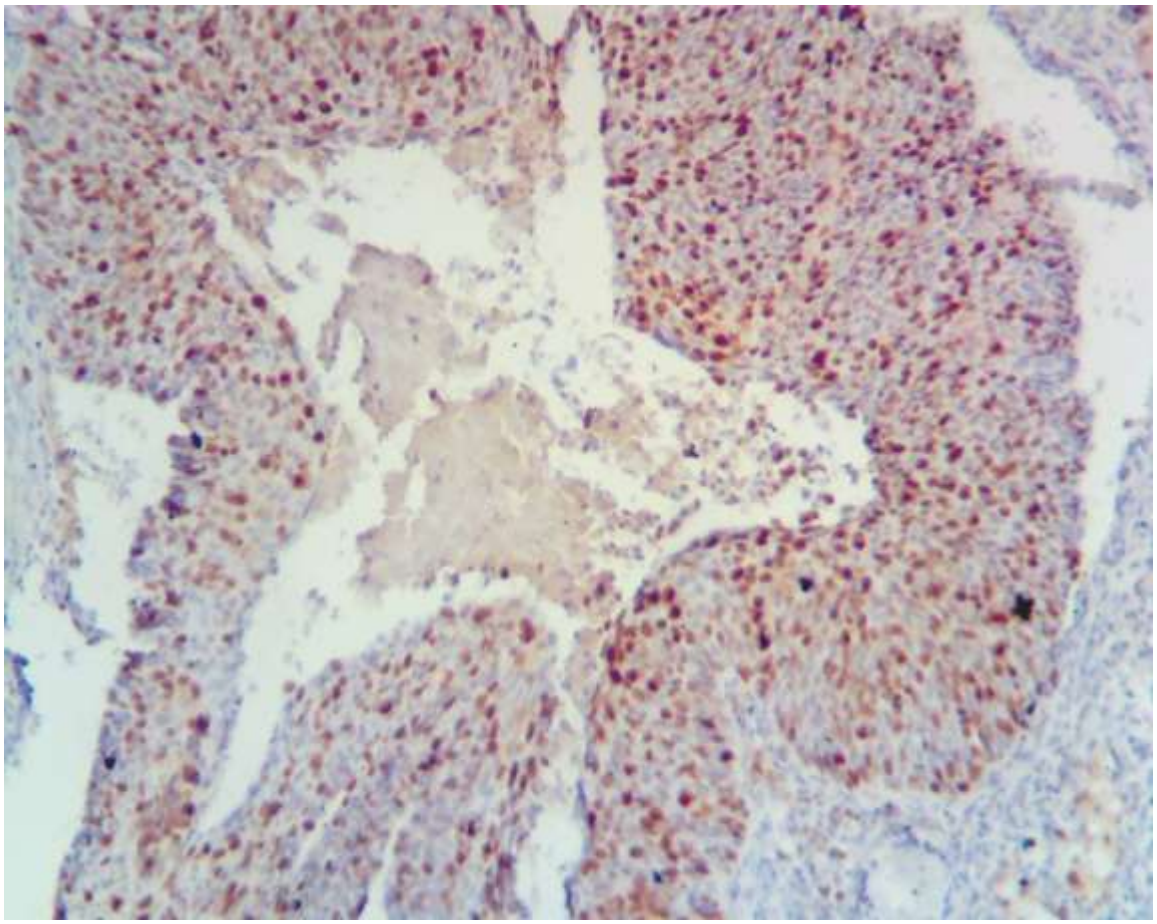

Fig.7. Photomicrograph showing 3+nuclear positivity of Ki-67 in a case of large cell non keratinising carcinoma

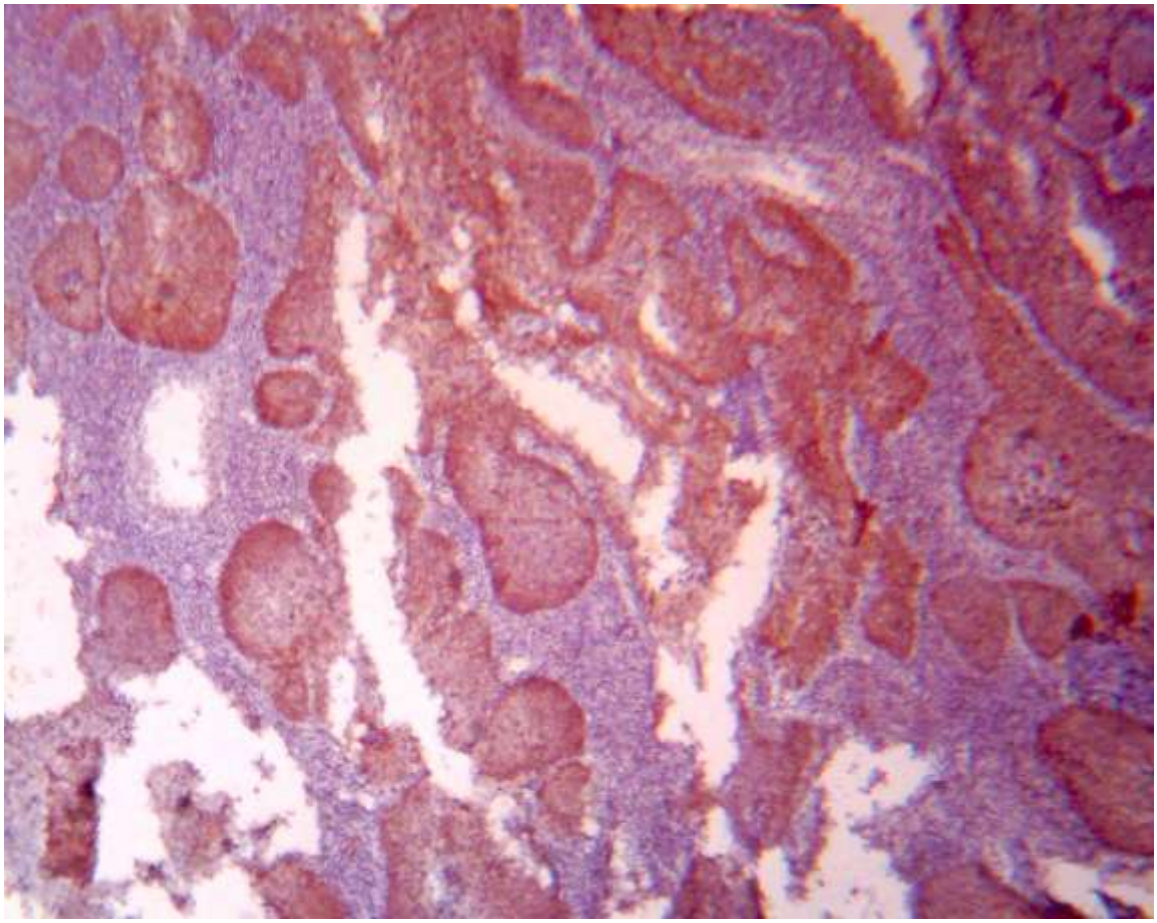

Fig. 8. Low power view of grade 3+ p16 positivity in large cell non keratinising carcinoma

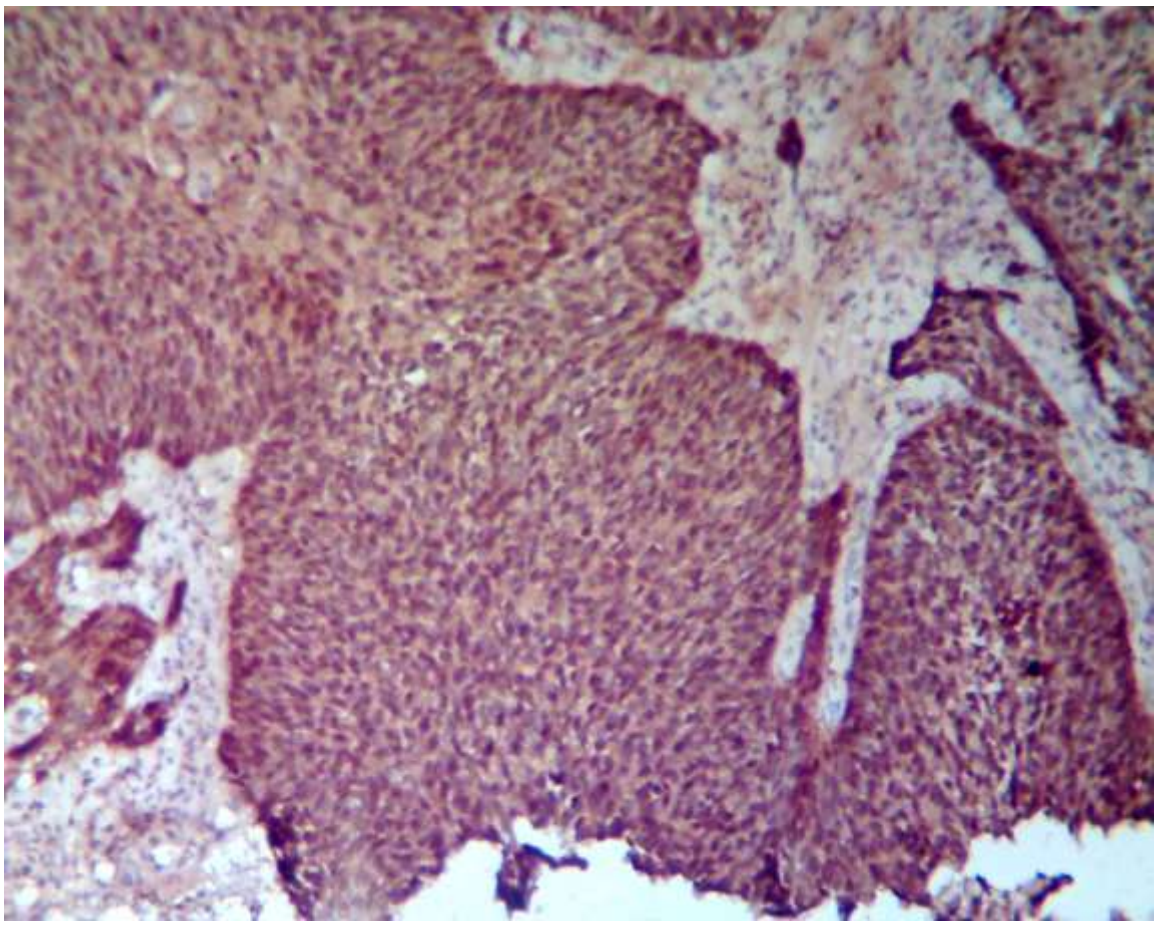

Fig. 9. Photomicrograph showing 3+nuclear and cytoplasmic positivity of p16 in a large cell non keratinising carcinoma

DISCUSSION

Carcinoma cervix remains the leading cause of deaths due to cancer in India. Much efforts have been taken to bring down the mortality through screening programmes at various levels of the health sectors. However as already mentioned, this is subjected to inter observer variation which can be overcome by the use of bio markers.

The screening programmes are based on the fact that neoplasia of the cervix is preceeded by a long period of slow progress through intra epithelial stages. Further the association of this cancer with an infectious agent, HPV has also been widely studied based on which they are categorised into low and high risk types. This helps in making use of the various bio markers to study the proliferative potential and the outcome of these lesions.

Ki-67 as well known is a proliferative marker that is expressed in all stages of the cell cycle except G0 phase.[62] It is used in various malignancies to study the aggressiveness so that appropriate treatment can be implemented. It is highly accurate in demonstrating the cervical lesions with high potential of turning into malignancy. Thus this marker is widely used to prognosticate dysplastic lesions of the cervix.

P16 is a tumor suppressor protein that is encoded by the *CDKN2A* gene. Its major role in cell cycle is down regulation of cell cycle by inhibiting the progression of cell cycle from G1 to S phase[63]. It has its effect expressed through the HPV genome. It has wide application in malignancies like squamous cell

carcinoma and melanoma. Since the majority of cervical malignancies reported come under the squamous variant, its use in these malignancies is definite.

With the studies available, the expression of Ki-67 and p16 were studied in both dysplastic and malignat lesions of the cervix to understand the prognosis of the same which cannot be done through histopathological examination though wide arrays of potential biomarkers have been evaluated for the diagnostic usefulness of cervical cancer and its precursors. These studies were mainly done to minimise the surgical intervention and to bring down the costs of repeated screening programmes.

In a study by C. H.Chi, MD et al., (1977), the distribution of mitotic figures which were studied revealed that the mitotic figures which were normally confined to the basal layer showed an increase in both frequency and distribution as the grade of dysplasia increases[64,65]. This study also showed an increase in mean mitotic count with increasing grades of dysplasia.

In the study by Srivastava S.et al. (2010),[66] the expression of MIB-I increased from normal cervical epithelia to varying severity of CINs to carcinoma. It was statistically significant when comparison was between controls and cases and the same was insignificant within the groups. MIB-I positivity was seen in 14 of 15 cases of CIN I, 15 of 15 cases of CIN II, 3 of 3 cases of CIN III and 15 of 15 cases of carcinoma cervix.

In this study also, 9 of 14 CIN-I, 5 of 8 CIN-II, 6 of 6 CIN-III cases, 20 of 21 carcinoma cases showed Ki-67 expression. The % expression also showed a constant increase from 64% in CIN-1 to 100% in malignant cases. However, one case of CIN-I showed grade III positivity which may indicate the lesion would progress vigorously.

In the same study, p16 INK4A overexpression was seen in all CIN I lesions (15/15), all CIN II lesions (15/15), all CIN III lesions (3/3) and all cases of carcinoma cervix (15/15) of tissue biopsies.

In a study by Klaes *et al*. (2001),[67] positive expression of p16 was found in all CIN I lesions (*n* = 47), all CIN II lesions (*n* = 32), all CIN III lesions (*n* = 60) and 56 of 58 invasive Squamous cell carcinoma (SCC). However this expression was found to be minimal in inflammatory and low grade dysplasias.

In the present study, p16 expression was very much less (4/14, i.e.21%) in CIN-1 compared to the above study .The expression in CIN II and CIN-III was also less (62% and 83%). However the expression in malignant lesions was similar to the above study.

In the same study by Srivastava S.et al. (2010), correlation between Ki-67 and p16 expression was, an increasing p16 expression with consistently increasing MIB-1 LI in the groups of increasing severity. In this study also, the correlation between the two markers was seen and it was also statistically significant.

Gupta et al. Gynecol Obstet 2013,[68] study which comprised of 20 cases of dysplasia and 30 cases of carcinomas revealed that the mean value of LI (Labelling Index) was found to increase as the nature of the lesion changed from dysplasia (16.94) to carcinoma (50.754) with the difference being found to be extremely statistically significant (p value <0.0001).

MIB-1 LI also showed an increase with increase in dysplasia from CIN-I (13) to carcinoma (45.1) in this study. Further, the correlation between the mean of mitotic count and that of the Mib-1 was also statistically significant.

CONCLUSION

- Ki-67 and p16 expression were seen in CIN-I, II, III and squamous cell carcinomas of the cervix with **statistically significant** correlation of expression between them.

- The mean mitotic count increases with increasing dysplasia which is an indirect measure of proliferative potential. It also showed **statistically significant correlation** with mean MIB-1 LI.

- Though all CIN-I cases showed a low Ki-67 positivity, one showed 3+ positivity. This implies that not all CIN-I cases have low malignant potential and a cautious follow up of such cases is mandatory.

- The scoring of expression of Ki-67 increases with increasing dysplasia of the lesion. However even high grade dyplasias showed 0/1+ positivity which indicates their low proliferative potential and hence the tumor progression may be slow in such cases which requires a long term follow up.

- p16 had a better expression in high grade intra epithelial lesions(60-80%) and malignancies (100%) of the cervix compared to low grade dysplasias (21%) which may be due to the integration of HPV with high grade lesions. Further LCNK variant showed a lesser positivity (70%) compared to LCK carcinomas (100%). This may be due to the lesser prevalence of HPV in this histological subtype.

- Adeno carcinoma did not show Ki-67/p16 positivity which had shown positive results in recent studies. But since only one case has been studied, this cannot be emphasised.

- To conclude, Ki-67 and p16 expression in pre malignant and malignant lesions of the Cervix can be used in conjunction with the histo morphological features to study their proliferative potential and thereby their progression which would have a major impact on the treatment.

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MASTER CHART FOR STUDY CASES

S. No	Age	I.P.No	Path. No	Diagnosis	Mitotic count (%)	Ki-67 expression	P16 expression grading	Mib-1 LI
1	62	30805/15	1180/15	LCNK	2.8	3+	0+	57
2	43	25152/15	1245/15	CIN-I	0.9	2+	0+	35
3	38	34626/15	1373/15	CIN-I	1.1	2+	0+	25
4	45	16336/15	668/15	CIN-II	1.3	3+	2+	38
5	43	257482/15	207/15	CIN-I	0.7	2+	1+	22
6	50	6478/15	320/15	LCK	2.3	3+	2+	42
7	41	239242/15	2973/14	CIN-I	0.8	0	0+	0
8	40	59902//14	3053/14	CIN-II	1.1	0+	0+	0
9	45	61054/14	3008/14	LCNK	2.5	3+	1+	45
10	45	14696/15	686/15	CIN-I	0.8	3+	0+	34
11	48	43271/14	3025/14	LCNK	3	3+	0+	59
12	70	26889/15	1053/15	LCNK	2.6	3+	3+	49
13	42	23225/15	1045/15	CIN-I	0.8	1+	2+	13
14	52	64062/14	3104/14	Adeno	0.9	0+	0+	0
15	60	59671/14	3005/14	LCK	1.7	3+	3+	62
16	70	22754/15	973/15	LCNK	2.3	0+	0+	0
17	55	17909/15	914/15	LCNK	2.9	3+	3+	52
18	75	17090/15	918/15	LCNK	1.8	2+	2+	39
19	75	23178/15	958/15	LCNK	2.3	3+	3+	48
20	70	28903/15	1011/15	LCK	3.2	2+	2+	35
21	55	62538/14	3080/14	LCK	2.7	3+	2+	47
22	30	13020/14	701/14	CIN-I	1.1	2+	0+	21
23	70	25270/14	1305/14	CIN-I	0.9	1+	0+	10
24	40	8328/15	336/15	CIN-III	2.5	1+	1+	23
25	53	38861/14	1930/14	LCK	2.9	3+	1+	66
26	51	4683/15	248/15	LCNK	3.1	3+	2+	59
27	60	1227/15	142/15	CIN-III	2.2	3+	1+	47

S. No	Age	I.P.No	Path. No	Diagnosis	Mitotic count (%)	Ki-67 expression	P16 expression grading	Mib-1 LI
28	59	6526/15	324/15	LCK	2.8	2+	3+	27
29	36	72932/15	35/15	CIN-I	1.3	2+	0+	22
30	65	888/15	147/15	LCNK	3.3	2+	2+	44
31	65	6815/15	284/15	CIN-III	1.2	2+	1+	48
32	30	220514/14	2254/14	CIN-II	0.9	0+	1+	0
33	60	8833/15	378/15	CIN-III	2.2	3+	2+	51
34	31	228180/14	2608/14	CIN-II	1.5	0+	0+	0
35	31	68664/13	2793/13	CIN-II	1.3	1+	0+	27
36	38	5443/15	87/15	CIN-III	2.7	3+	0+	35
37	55	34823/14	2081/14	CIN-I	1	0+	1+	0
38	35	37347/14	1880/14	CIN-I	0.7	0+	0+	0
39	49	19980/15	802/15	LCNK	2.6	3+	3+	43
40	50	45030/14	2249/14	CIN-I	0.6	0+	0+	0
41	51	40576/10	2032/14	CIN-I	0.5	1+	0+	0
42	72	60596/14	3071/14	LCNK	2	3+	0+	57
43	30	9253/15	385/15	LCK	3.1	2+	2+	31
44	72	62509/14	3099/14	CIN-II	1.2	1+	2+	21
45	70	62972/14	3063/14	LCK	1.9	3+	3+	49
46	35	9235/15	381/15	CIN-II	0.9	1+	3+	27
47	43	4458/15	345/15	CIN-III	1.1	1+	2+	43
48	36	179052/15	366/15	CIN-I	1	0+	0+	0
49	47	4716/15	243/15	LCNK	3.2	3+	1+	39
50	44	45851/14	2385/14	CIN-II	2.1	1+	1+	13

ANNEXURE - I

WHO CLASSIFICATION OF CERVICAL TUMORS

EPITHELIAL TUMORS:

Squamous tumors and precursors

Squamous cell carcinoma, not otherwise specified

- Keratinising
- Non keratinising
- Basaloid
- Verrucous
- Warty
- Papillary
- Lymphoepithelioma
- Squamotransistional

Early invasive (microinvasive) squamous cell carcinoma

Squamous intraepithelial neoplasia

- Cervical intraepithelial neoplasia(CIN3)
- Squamous cell carcinoma in situ

Benign squamous cell lesions

- Condyloma accuminatum
- Fibroepithelial polyp

Glandular tumors and precursors

Adenocarcinoma

- Mucinous adenocarcinoma
- Endocervical
- Intestinal
- Signet-ring cell
- Minimal deviation
- Villoglandular
- Endometrioid adenocarcinoma
- Clear cell adenocarcinoma
- Serous adenocarcinoma
- Mesonephric adenocarcinoma

Early invasive adenocarcinoma

Adenocarcinoma in situ

Glandular dysplasia

Benign glandular lesions

- Mullerian papilloma
- Endocervical polyp

Other epithelial tumors

- Adenosquamous carcinoma
- Glassy cell carcinoma variant
- Adenoid cystic carcinoma
- Adenoid basal carcinoma
- Neuroendocrine tumors

- Carcinoid
- Atypical carcinoid
- Small cell carcinoma
- Large cell neuroendocrine carcinoma
- Undifferentiated carcinoma

MESENCHYMAL TUMORS AND TUMOR LIKE CONDITIONS

- Leiomyosarcoma
- Endometrioid stromal sarcoma, low grade
- Undifferentiated endocervical sarcoma
- Sarcoma botryoides
- Alveolar soft part sarcoma
- Angiosarcoma
- Malignant peripheral nerve sheath tumor
- Leiomyoma
- Genital rhabdomyoma
- Postperative spindle cell nodule

MIXED EPITHELIAL AND MESENHYMAL LESIONS

- Carcinomasarcoma
- Adenosarcoma
- Wilms tumor
- Adenofibroma
- Adenomyoma
- Melanocytic tumors

MISCELLANEOUS TUMORS

- Tumors of germ cell type
- Yolk sac tumor
- Dermoid cyst
- Mature cystic teratoma
- Lymphoid hematopoeitic tumors
- Secondary tumors

ANNEXURE - II

Staging is done by FIGO staging (94)

Stage I	Cervical carcinoma confined to uterus (extension to the corpus should be disregarded)
Stage IA	Invasive carcinoma diagnosed only by microscopy; all macroscopically visible lesions, even with superficial invasion, are stage IB
Stage IA1	Stromal invasion no greater than 3.0 mm in depth and 7.0 mm or less in horizontal spread
Stage IA2	Stromal invasion more than 3.0 mm and not more than 5.0 mm with a horizontal spread of 7.0 mm or less
Stage IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than IA2
Stage IB1	Clinically visible lesion 4.0 cm or less in greatest dimension
Stage IB2	Clinically visible lesion more than 4.0 cm in greatest dimension
Stage II	Tumor invades beyond the uterus but not to pelvic wall or to lower third of the vagina
Stage IIA	Without parametrial invasion
Stage IIA1	Clinically visible lesion _4.0 cm in greatestdimension
Stage IIA2	Clinically visible lesion >4 cm in greatest dimension
Stage IIB	With parametrial invasion
Stage III	Tumor extends to the pelvic wall and/or involves lower third of vagina and/or causes hydronephrosis or nonfunctioning kidney
Stage IIIA	Tumor involves lower third of vagina with no extension to pelvic wall
Stage IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or nonfunctioning kidney
Stage IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum.
Stage IVA	Spread of the growth to adjacent organs
Stage IVB	Spread to distant organs